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The Pharmacognosy of the Periwinkles: Vinca and Catharanthus

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Interest in the group of plants known as the Periwinkles has increased during the past few years. This interest has resulted largely because of the isolation of vincaleukoblastine from one of the members of this group, *Catharanthus roseus*, by Noble, Beer and Cutts in 1958 (1). This alkaloid has been evaluated clinically and is currently being used in the treatment of Hodgkin's disease and choriocarcinoma (2,3). This discovery has prompted further investigation into other plants in the Periwinkle group with the hope of finding additional sources of vincaleukoblastine or congeners having either lesser toxicity or greater activity.

The two genera *Vinca* and *Catharanthus* comprise the group of plants commonly referred to as the Periwinkles, however, much confusion exists concerning proper nomenclature within these genera. This confusion has led to some difficulty in the interpretation of published data on the chemistry of these plants. Acknowledging the existence of such confusion, an attempt will be made to review the literature pertaining to the Periwinkles using current taxonomic concepts to include morphological and anatomical features of these plants, folk uses, phytochemistry, chemotaxonomy and pharmacology.

The Periwinkles are members of the alkaloid-rich Apocynaceae which has been discussed in the several recent review papers concerned with this plant family. The paper by Bisset (4) represents the most comprehensive review of the Apocynaceae available today, and is primarily concerned with the alkaloids of this group. It is the first report in the literature which compares the alkaloid content to apocynaceous plants using the taxonomic classification of Pichon (5,6). An attempt will be made in later portions of this paper to show that there is excellent agreement between this taxonomic classification and the phytochemicals isolated from species within this group. Bisset has reviewed 60 genera in the family and has cited some 600 references. Willaman and Schubert (7) have reported on the literature citations concerning alkaloids of the Apocynaceae in a survey of data reported through 1955 and including some references from 1956. The most recent compilation of alkaloid data from the Apocynaceae has been published by Raffauf and Flagler (8) in which an attempt was made to correlate alkaloid types with the taxonomy of this group. Although these authors readily admitted that no definite conclusions could be drawn in respect to alkaloid chemotaxonomy of the Apocynaceae, it could be noted that excellent correlation does exist within certain

groups. Fairly complete physical data were cited for some 181 different alkaloids and notations were made concerning others.

More specific review articles concerning the Periwinkles have been published by Paris (9), Schulz (10), Gabbai (11) and Paris and Moyse (12–14).

CLASSIFICATION OF THE PERIWINKLES

The genus *Vinca* was originally established by Linnaeus in 1735 (15). *V. minor, V. major, V. rosea, V. lutea, V. pusilla, V. difformis, V. herbacea* and *V. pubescens* were subsequently described as appropriate to this genus.

In 1828 Reichenbach added the genus *Lochnera* to the Apocynaceae and included one species, *Lochnera rosea* (*V. rosea*). In August of 1838 Endlicher described the differences between the genera *Vinca* and *Lochnera* but previously, in February of the same year, G. Don (16) had established the genus *Catharanthus*, the general characteristics of which were identical to those of the proposed genus *Lochnera*. Don described the characteristics of *Catharanthus roseus* and *Catharanthus pusillus*. Since the publication of Don antedates that of Endlicher, the genus *Catharanthus* has priority over *Lochnera*.

Pichon (5,6) has recognized this in his recent work on the classification of the Apocynaceae in which he has classified *Vinca* and *Catharanthus* as follows:

Family: Apocynaceae
Sub-family: Plumerioideae
Tribe: Alstonieae
Sub-tribe: Catharanthinae
Genus: *Catharanthus* G. Don

Section 1. *Lochnera* (Reichb. f.) Pich.
Catharanthus lanceus (Boj. ex A. DC.) Pich.
Catharanthus longifolius (Pich.) Pich.
Catharanthus trichophyllus (Bak.) Pich.
Catharanthus roseus (L.) G. Don

Section 2. *Cupa-veela* (A. DC.) Pich.
Catharanthus pusillus (Murr.) G. Don

Section 3. *Androyella* (Pich.) Pich.
Catharanthus scitulus (Pich.) Pich.

Vinca L.
Vinca herbacea Waldst et Kit.
var. *libanotica* (Zucc.) Pich.
var. *sessilifolia* (A. DC.) Pich.
var. *herbacea*
Vinca major L.
var. *difformis* (Pourr.) Pich.
var. *major* Pich.
Vinca minor L.

BOTANICAL CONSIDERATIONS

Morphology, Synonymy and Distribution of the Species of Catharanthus.—Pichon (5) considers the genus *Catharanthus* to comprise six species of small shrubs and herbs which are predominantly indigenous to Madagascar.

Catharanthus roseus (L.) G. Don Fig. 1.

C. roseus (*Vinca rosea* L., *Lochnera rosea* (L.) Reichb. f.) is a pantropical species, probably originating in Madagascar and spreading to India, Indochina, Indonesia, Philippines, Australia, South Africa and the Orient, North America, the West Indies, Guiana, Brazil and cultivated in Europe and other countries as an ornamental. It is a fast growing sub-shrub, woody at the base, 40–80 cm high with

erect branches. The leaves are opposite, oblong and petiolate (3–8 cm × 1.5–5 cm) having an acute base and round or mucronate tip. The plant is pubescent, especially when young, but a glabrous variant does exist. The flowers are violet, rose or white but ocellate forms are often found in cultivated varieties. Two forms of *C. roseus* (normally a violet or rose colored flowering plant) are recognized. These are f. *albus* (Sweet) G. Don with a white corolla and f. *ocellatus* (Sweet) G. Don with a pink or red eye in the otherwise white corolla.[1] The follicles are 2.5–4 cm long and 2–3 mm in diameter containing from 12–20 seeds, each with a thin black tegumen (12).

Catharanthus lanceus (Boj. ex A. DC.) Pich. Fig. 1.

C. lanceus (*Lochnera rosea* Boj. ex A. DC., *Vinca lancea* (Boj. ex A. DC.) K. Schum.) is indigenous to Madagascar. It is a perennial herb with a sub-woody base and smooth quadrangular stem, attaining a height of from 30 cm to 1 m. The leaves are oblong, sub-sessile and from 1.5–2.5 cm long and 0.5–1 cm wide. The flowers are violet or rose colored, having a linear lobed calyx. It is most frequently found growing on the high plateaus of Madagascar. Plants found growing on rocks and dry slopes often exhibit ericoid leaves (12).

Catharanthus trichophyllus (Bak.) Pich. Fig. 1.

This species, also known as *Lochnera trichophylla* Pich., is found growing on the East coast of Madagascar as well as in stony areas of Isalo. It is a bushy plant having a woody base and quadrangular stem reaching a height of 50 cm to 1 m. The leaves are similar in size to those of *C. roseus* but are sessile and have a pointed apex in addition to possessing hairs on the veins. Glabrous forms are also known to exist. The flowers are rose to red and the follicles are 5 cm long (12).

Catharanthus longifolius (Pich.) Pich. Fig. 3.

C. longifolius (*Lochnera longifolia* Pich.) is a sub-shrub of from 40 cm to 1.5 m high with a quadrangular stem. The leaf (4–9 cm × 0.3–0.9 cm) is short-petiolate, pubescent and linear-lanceolate with an angular base. The flowers are purple. The plant is found growing in the central and southern parts of Madagascar and is cultivated in the garden of Tsimbazara (12).

Catharanthus pusillus (Murr.) G. Don Fig. 3.

C. pusillus (*Vinca pusilla* Murr., *Lochnera pusilla* (Murr.) K. Schum.) is an annual herb indigenous to India, having a straight, smooth, quadrangular stem and attaining a height of 60 cm. The leaves are opposite and acuminate (5–6 cm × 1–2 cm). Flowers are small and white with divergent, small, membranous follicles. This species of *Catharanthus* is unique in being the only member of the genus not indigenous to Madagascar (12).

Catharanthus scitulus (Pich.) Pich. Fig. 1.

This species is a very small annual herb, 3–20 cm in height and having smooth, oblong leaves (0.7–1.5 cm × 0.2–0.6 cm). It is reported as growing only in the southwestern parts of Madagascar. The only synonym found for this species is *Lochnera scitula* Pich. (5,12).

Morphology, Synonymy and Distribution of the Species of Vinca.—Close to 40 species of *Vinca* have been described at various times in the literature. Many of these have been demonstrated to be identical while others have been assigned to the genus *Catharanthus*. Pichon (6) has pointed out that many of these are transition forms and reduces them to three distinct species.

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[1]Several other flower colors occur as horticultural variants and seeds for these plants are available from a number of commercial sources.

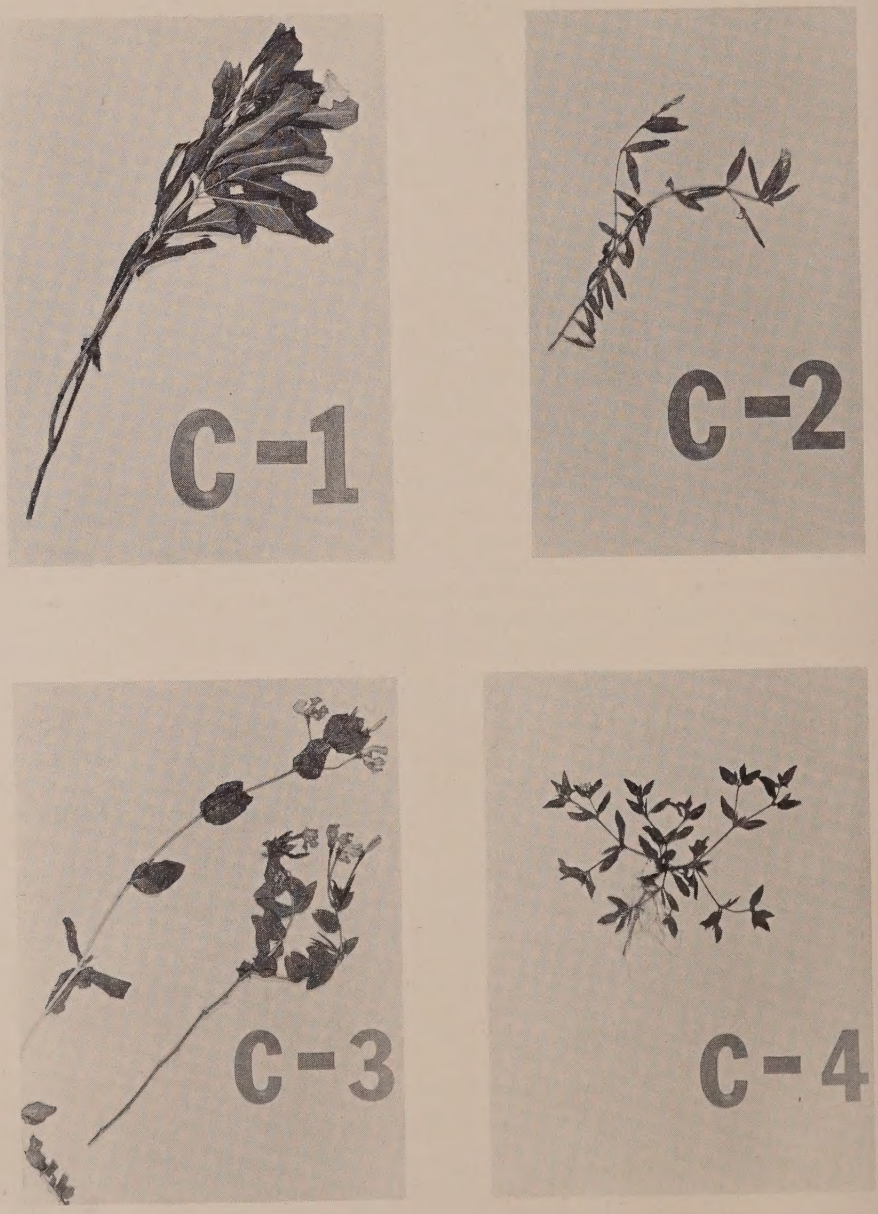

FIG. 1. *Herbarium specimens of Catharanthus species.*
(C–1) Catharanthus roseus (L.) G. Don. (Lebanon—1956).
(C–2) Catharanthus lanceus (Boj. ex A. DC.) Pich. (Tananarive—1956).
(C–3) Catharanthus trichophyllus (Bak.) Pich. (Isalo—1959).
(C–4) Catharanthus scitulus (Pich.) Pich. (Madagascar—1943).
Scale: Height of C is 38 mm.

Vinca herbacea Waldst. et Kit.

Pichon recognizes three varieties, each with analogous low growth, seldom erect, with blue-violet flowers and oblong, soft leaves and uneven margins.

var. **libanotica** (Zucc.) Pich. Fig. 3.

This variety is indigenous to Turkestan, Syria, Lebanon, Cilicia and Mesopotamia. It is synonymous with *V. libanotica* Zucc., *Vinca Bottae* Jaub. et Spach., *Vinca erecta* Regel et Schmalh. and *Vinca herbacea* Waldst. et Kit. var. *glaberrima* A. DC. The leaves of this variety of *V. herbacea* do not have indented margins (6,12).

var. **sessilifolia** (A. DC.) Pich. Fig. 3.

This variety is reported only in Turkish Armenia and is synonymous with *Vinca sessilifolia* A. DC. and *Vinca Haussknechtii* Bornm. et Sint. The leaves are larger (3–7 cm long) than other varieties and have an entire margin (6,12).

var. **herbacea** Pich. Fig. 2.

This variety of *V. herbacea* includes *Vinca pumila* Clarke, *Vinca mixta* (Velen.) Velen. and *Vinca erecta* Regel et Schmalh. var. *bucharica* B. Fedtsch. It is indigenous to southern Europe (lower Austria), through the Middle East, and southern Russia as far south as Turkestan. The leaves have rough margins, rarely ciliate, and are from 2 to 5 cm long (6,12).

Vinca major L.

V. major exhibits a dimorphic growth; sterile creeping and erect flowering branches. The leaf is soft and obovate. Pichon (6) describes two varieties.

var. **major** Fig. 2.

V. major var. *major* has been known by a number of synonyms. These include: *Pervinca major* (L.) Garsault, *Vinca difformis* Pourr., *Vinca grandiflora* Salisb., *Vinca media* Hoffmgg. et Link, *Vinca ovatifolia* Stokes, *Vinca pubescens* Urv, *Vinca acutiflora* Bertol., *Vinca intermedia* Tausch, *Vinca obliqua* Porta, *Pervinca media* (Hoffmgg. et Link) Caruel, *Vinca obtusiflora* Pau and *Vinca lusitanica* Brot. It has a curiously disjunct distribution in France, Italy, Switzerland, Sicily, Madeira, Canary Islands, Crete, Rhodes, Chios, South and East shores of the Black sea and probably introduced to England, Portugal, Spain, Malta, Algeria and the United States. This variety is differentiated from var. *difformis* chiefly in the well ciliated margins of the leaves and sepals (6,12).

var. difformis (Pourr.) Pich. Fig. 2.

This variety is also known as *Vinca difformis* Pourr., *Vinca media* Hoffmgg. et Link, *Vinca acutiflora* Bertol., *Vinca intermedia* Tausch., *Pervinca media* (Hoffmgg. et Link) Caruel., *Vinca obtusiflora* Pau. and *Vinca major* L. var. *glabra* F. Schultz. It is indigenous to the Azores, Spain, Portugal, France, Italy, Corsica, Sardinia, Balearic Islands, Morocco and Algeria. It differs chiefly in the shape of the leaf and sepals which are devoid of marginal hairs (6,12).

Vinca minor L. Fig. 2.

V. minor is commonly seen from western Europe to Rumania and in parts of european Russia, as well as in the United States where it is frequently utilized as a ground cover. It has been known as *Pervinca minor* (L.) Garsault, *Pervinca procumbens* Gilib., *Vinca humilis* Salib. and *Vinca ellipticifolia* Stokes. It also exhibits a dimorphic growth with a sterile creeping type of branch and an erect flower-bearing type. The leaves are elliptical, firm, smooth and have an entire

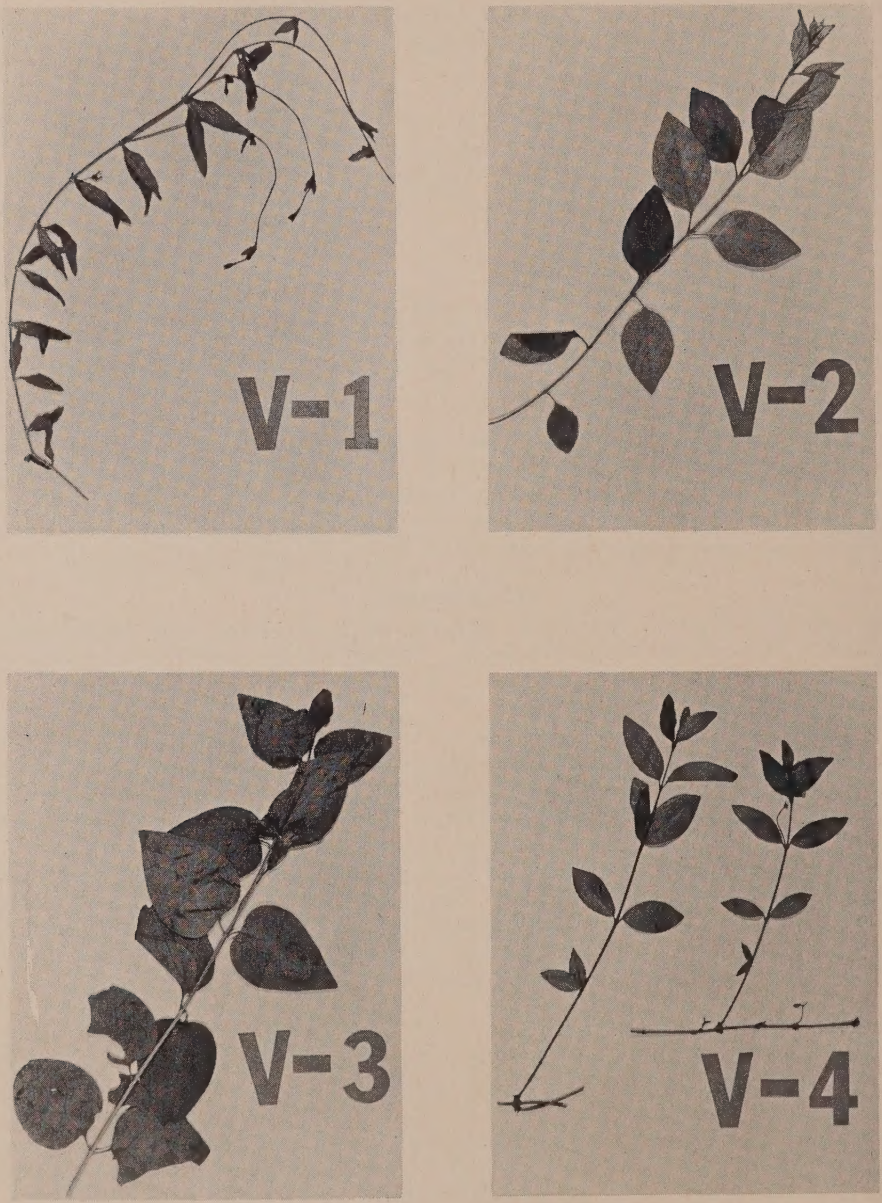

FIG. 2. *Herbarium specimens of Vinca species.*
(V-1) Vinca herbacea Waldst. et Kit. var. herbacea. (Jardin alpin du Museum de Paris—1956).
(V-2) Vinca major L. var. major Pich. (Paris—1956).
(V-3) Vinca major L. var. difformis (Pourr.) Pich. (Hyeres-Var, France—1961).
(V-4) Vinca minor L. (Pittsburgh, Pennsylvania—1961).
Scale: Height of V is 38 mm.

FIG. 3. *Herbarium specimens of Vinca and Catharanthus species.*
(C–5) Catharanthus longifolius (Pich.) Pich. (Madagascar—1933).
(C–6) Catharanthus pusillus (Murr.) G. Don (India).
(V–5) Vinca herbacea Waldst. et Kit. var. libanotica (Zucc.) Pich. (Mesopotamia).
(V–6) Vinca herbacea Waldst. et Kit. var. sessilifolia (A. DC.) Pich. (Turkish Armenia).
Scale: Height of C and V is 38 mm.

margin (4–6 cm × 1.5–2 cm). The typical flower is blue but white flowering variants exist as well as horticultural forms (17,127).

Anatomy of Vinca and Catharanthus.—Very little has been reported relative to the anatomy of the Periwinkles. Perhaps the most comprehensive, yet incomplete, reports have been published by Gabbai (11) and Paris and Moyse (12). These investigators have compared the anatomy of the leaves, stems and roots of several *Vinca* and *Catharanthus* species but the material investigated was not uniform with respect to the age, geographic distribution or ecological conditions of the plants studied. This has resulted in a comparison of species which may not be entirely valid. The anatomy of *V. minor* and *V. major* var. *major* leaves has also been briefly reported by Planchon and Collin (18) while Jean (19) has included an intensive study of the internal phloem of young *V. major* var. *major* plants in a comparative study of selected families of dicotyledons. Pannocchia-Laj (20) has studied the embryology and karyology of *V. minor*, *V. major* var. *major* and *V. major* var. *difformis* while Finn (21) has reported on the anatomy of the sperm cells of *V. minor* and *V. herbacea* var. *herbacea*.

From the incomplete data reported in the literature concerning the anatomy of the Periwinkles it can be generalized that there are few differentiating characteristics of diagnostic value between the genera *Vinca* and *Catharanthus*. Perhaps one of the causes of variation in some of these diagnostic features is ecological in nature. For example, young *C. roseus* plants usually are pubescent but not always so (11), non-flowering plants (*V. major* var. *major*) possess a greater number of sieve tubes and companion cells in the stem phloem than flowering plants and the cells are usually larger. Some of the phloem cells in the flowering stems of many plants are crushed. The cambium in the flowering stem becomes less active and phloem parenchyma instead of sieve tubes and companion cells is formed. There is more callose in the phloem cells of the flowering than in the non-flowering stem (22). The leaf cuticle of *Vinca* plants is usually thicker than the cuticle of *Catharanthus* plants but this has been ascribed to climatic conditions (11).

Genetic Studies of the Periwinkles.—Colchicine-induced tetraploid *C. roseus* has been shown to exhibit thicker stems, thicker leaves and larger flowers (23–26). In studies concerned with the inheritance of flower color in *C. roseus*, Flory (27) found that the red pigmentation in stems is associated with flower color and although the inheritance of this pigmentation character was not analyzed, the factor responsible for it appears to be closely linked with the genes that account for flower color. Red color is associated with the stems of all pink flowering plants and also, in lesser amounts, in all white red-eyed ones. Such pigmentation has not been found in the stems of white flowering plants.

The chromosome numbers for several *Vinca* and *Catharanthus* species have been recorded (28) and in view of the more recent taxonomic classification of these plants, the list should be completed.

Tissue Culture Methods for the Periwinkles.—The growth of single cell cultures of *C. roseus* has been reported by Muir *et al.* (29) and the influence of various carbon compounds on the growth of this same plant in tissue culture has also been described (30). Reports concerning the tissue culture of other Periwinkles have not been found in the literature.

THE PERIWINKLES AS FOLK REMEDIES

Many of the Periwinkles have a long history of alleged usefulness in treating a variety of illnesses. For the most part, the activity can be accounted for by constituents that are known to be present in these plants. For example, *V. minor* leaf has been used as a vomitive (31) and it is not unreasonable to assume that this activity is brought about by the irritant triterpenoid saponin ursolic acid which is known to be present in the leaves (32–36). Perhaps the hemostatic (31) and astringent (38) activity of *V. minor* is brought about by the tannin con-

tent (39,40). The dried leaves of this same plant have also induced hypotension when taken orally in daily doses of 3 g (37). Recent clinical experience has shown that either the alkaloid vincamine (41–43) or a mixture of alkaloids (44) is responsible for this activity. Perhaps the most unexplainable folk use for any of the Periwinkles is the anti-diabetic activity of the leaves of *C. roseus* when used in the form of a tea by the people of South Africa, Natal, Australia, South Vietnam, the Philippines and England (1,45–50). Proprietary preparations are available in South Africa ("Covinca") and in England ("Vinculin") which are essentially infusions of the leaves of *C. roseus* (*V. rosea*) (1,45). Epstein (51), Lee and Drew (52) and Garcia (53) have attempted to produce hypoglycemia in rabbits with extracts of *C. roseus* leaves. The latter investigator was able to show some degree of hypoglycemic activity in his experiments but the number of animals in the experiment was too small to draw valid conclusions. Epstein and Lee and Drew were not able to substantiate these results using larger numbers of animals. Clinical trials of a *C. roseus* leaf tincture in 5 diabetic patients was reported by Nye and Fitzgerald in 1928 (54). These investigators could not demonstrate appreciable hypoglycemic effects. Corkill and Doutch (55) utilized an aqueous infusion of *C. roseus* leaves in treating 15 diabetic patients. These workers concluded that the preparation acted as an ideal purgative in those patients suffering from constipation but did not exert hypoglycemic activity.

An important factor to consider in evaluating folk remedies is correct identification of the plant material actually used. This has been pointed out by Cowley and Bennett (46) who have questioned the statement by Martindale (56) in regard to *V. major* var. *major*, grown in Natal, being used for its anti-diabetic properties. Cowley and Bennett are of the opinion that the plant mentioned by Martindale was actually *Catharanthus roseus* since *V. major* var. *major* is not reported as growing in Natal.

The many types of activity attributed to the Periwinkles as home remedies are listed in table 1 together with the part(s) of the plant used, the particular species employed and the general geographic area involved. The method of preparation in most cases consisted of extraction with water either with or without heat. References cited in the table will provide this information in more detail.

PHYTOCHEMISTRY

Alkaloid occurrence and distribution.—All of the Periwinkles that have been chemically investigated to date give positive tests for alkaloids. Only *C. scitulus* and *V. herbacea* var. *sessilifolia* have been overlooked in regard to chemical investigation. Although no crystalline alkaloids have been isolated from *C. pusillus*, *C. longifolius*, *C. trichophyllus* and *V. herbacea* var. *herbacea*, reports indicate their presence in these plants. Vintilesco and Ioanid (66) have reported that *V. herbacea* var. *herbacea* contains an alkaloid substance with chemical properties similar to strychnine while Bocharova (67) has isolated an alkaloid fraction from this plant having atropine-like activity. Zolotnitskaya (68), in a screening of Armenian plants, pointed out that *V. herbacea* var. *herbacea* was rich in alkaloids and crude alkaloid yields have been reported by Bocharova (67) from plants collected in the northern Caucasus. The aboveground parts at flowering yielded 0.47 per cent while the roots at fall harvest gave 1.5 per cent alkaloid. Paris and Moyse (69), however, indicate that the above ground parts of this same species and variety yield 0.75 per cent of crude alkaloids when collected in France and by electrophoresis show the presence of at least six distinct alkaloids.

The literature concerning the alkaloid content of various Periwinkles is quite extensive but for the most part is probably of little use in making valid comparisons of the true alkaloid accumulation in these plants. Rarely do any two investigators utilize the same procedure for isolating the alkaloids, plants are collected at different times of the year and from different geographic and climatic areas and

TABLE 1. *Folk uses of the Periwinkles.*

Species	Reported use	Part used[a]	Locality	References
Catharanthus roseus	Abortive agent	rt	Philippines	57,58
	Anti-diabetic	lf,wp	South Africa, Natal, India, Australia, South Vietnam, Philippines	45–50, 58–60
	Anti-galactagogue	lf,wp	France, South Vietnam	18,50,59
	Astringent	lf,rt,wp	South Vietnam	50,59
	Bitter	wp	South Vietnam	50,59
	Depurative (purgative)	rt,wp	Madagascar	50,59,61
	Diaphoretic	wp	South Vietnam	50,59
	Dysentery	rt,wp	South Vietnam	18,50,59
	Dyspepsia	lf	South Vietnam	18,50,59
	Emmenagogue	rt	Philippines	57,58
	Hemostatic	rt	Madagascar	61
	Indigestion	lf,wp	South Vietnam	18,50
	Intermittent fever (malaria)	rt,wp	South Vietnam Central Vietnam	31,50
	Menorrhagia	lf,rt	South Africa, India	47,60
	Menstrual regulator	ap	North Vietnam China	50
	Skin infections	lf,rt,wp	South Vietnam	50
	Toothache	rt	Madagascar	31,61
	Urinary antiseptic	ap	Vietnam	50
	Vermifuge	rt	Madagascar	61
	Vomitive	lf	Madagascar	61
	Vulnerary	wp,rt	Maurice	50,59
	Wasp stings	jp	India	60
Catharanthus lanceus	Astringent	lf	South Africa	48
	Bitter	lf	South Africa	48
	Dysentery	wp	Madagascar	62
	Emetic	lf	South Africa	48
	Galactagogue	ap	Madagascar	62
	Purgative	rt	Madagascar	62
	Vermifuge	rt	Madagascar	62
	Vomitive	ap	Madagascar	62
Catharanthus pusillus	Lumbago (external)	wp	India	63
Vinca minor	Anti-galactagogue	lf	France	18
	Astringent	lf	Europe	38
	Cure-all	lf	France	31,39
	Depurative (purgative)	rt	Madagascar	31
	Hemostatic	rt,lf	Madagascar Europe	31,64
	Hypotensive	lf	Poland	37
	Menorrhagia	lf	Europe	64
	Toothache	rt	Madagascar	31
	Vermifuge	rt	Madagascar	31
	Vomitive	lf	Madagascar	31
	Vulnerary	lf	Europe	64
	Wasp stings	jl	Oussa	31,65
Vinca major var. *major*	Abortifacient	wp	France	38,47
	Anti-galactagogue	wp	France	18,185
	Anti-hemorrhagic	wp	France	185
	Astringent	lf	South Africa France	31,47, 56,185
	Bitter	wp	France	185
	Diabetes[b]	lf	Natal	47
	Menorrhagia	lf	South Africa France	47,56 64
	Tonic	wp	France	185
	Vulnerary	wp	France	185

[a] *rt*, roots; *lf*, leaves; *wp*, whole plant; *ap*, aboveground parts; *jp*, juice of the plant; *jl*, juice of the leaves.

[b] Probably not *V. major* var. *major*, but *C. roseus*. See (46).

quite often the part of the plant utilized in the investigation is not identified. An even greater problem is the apparent disregard for proper botanical identification of the plant investigated and the availability of proper herbarium specimens for reference purposes. Noble *et al.* (1) have noted that *C. roseus* plants grown as annuals in England contain only about one-fourth as much vincaleukoblastine as plants collected as perennials growing in the West Indies. In addition, they

TABLE 2. *Crude alkaloid yield from species of Catharanthus*

Species	Part[a]	Habitat	Per cent	Reference
C. roseus	lf	Australia	0.85	46
C. roseus (smooth)	lf	India	0.60–0.67	112
C. roseus (smooth)	ls	Madagascar	0.05–0.10	122
C. roseus (hairy)	ls	Madagascar	0.12–0.55	122
C. roseus f. *albus* (smooth)	lf	Australia	0.59–0.62	46
C. roseus f. *albus* (smooth)	lf[b]	Australia	0.15	46
C. roseus (hairy)	ls	Madagascar	0.16	69
C. roseus	ls	Madagascar	0.50	100
C. roseus	ls	Madagascar	0.55	62
C. roseus	st	India	0.26–0.31	112
C. roseus f. *albus* (smooth)	st	Australia	0.20	46
C. roseus f. *ocellatus* (hairy)	lf	Australia	0.54	46
C. roseus	fl	India	0.005–0.007	112
C. roseus	fr	Madagascar	0.40	100
C. roseus	rt	Travancore	1.22	111
C. roseus	rt	Madras	1.18	111
C. roseus	rt	Indo-China	0.63	69
C. roseus	rt	India	0.78–0.85	112
C. roseus	rt	Madagascar	0.56	69
C. roseus (smooth)	rt	Madagascar	0.15	122
C. roseus (hairy)	rt	Madagascar	0.52	69
C. roseus (hairy)	rt	Madagascar	0.40	122
C. roseus	rt	Madagascar	1.0	100
C. roseus	rt	Guadeloupe	0.22	69
C. roseus	rb	Madagascar	2.50	100
C. roseus	rb	Trivandrum	9.00	106
C. roseus f. *albus*	rb	Trivandrum	4.50	106
C. lanceus	rt	Madagascar	0.20–0.30	62
C. lanceus	rt	Madagascar	0.34	122
C. lanceus	rt	Madagascar	1.4	97
C. lanceus	rt	Madagascar	0.63	69
C. lanceus	rt	Madagascar	1.3	96
C. lanceus	ls	Madagascar	0.10–0.15	122
C. lanceus	ls	Madagascar	0.55	96
C. lanceus	ls	Madagascar	0.21	69
C. lanceus	st	Madagascar	0.10–0.15	62
C. longifolius	wp	Madagascar	0.22	122
C. longifolius	lf	Madagascar	0.10	69
C. trichophyllus	wp	Madagascar	0.70	69
C. trichophyllus	ls	Madagascar	0.13	122
C. trichophyllus	rt	Madagascar	0.20	122

[a]Dry material unless otherwise noted: *lf*, leaves; *st*, stems; *ls*, leaves and stems; *rb*, root bark; *rt*, roots; *wp*, whole plants; *fl*, flowers; *fr*, fruits.
[b]Fresh material analyzed.

found that leukopenic activity was present in the roots, stems and leaves of this plant but not in the seeds and that the greatest activity was concentrated in the leaves. With the knowledge of this obvious variability of alkaloid accumulation in the Periwinkles because of different conditions of growth, ecology, methods of assay and the possibility of chemical variants of distinct species, it would appear

that little would be gained by discussing this data. However, a summary of this data is presented in table 2 and in table 3.

A few remarkable differences exist in comparing some of the results in these tables. For example, Pillay *et al.* (106) in 1957 have investigated *C. roseus* and *C. roseus* f. *albus* plants for the alkaloids in the root bark. It was determined that the root bark of the pink flowering plants yielded about 9.0 per cent of total alkaloids while the pink flowering plants only about half of this amount, the deficiency being mostly in the strong serpentine-like bases. It is interesting to note that the stem of pink flowering plants usually has a pink pigmentation, the pigment being roughly proportional to the amount of red or pink pigment in the flower. White flowering plants are usually devoid of this pigment in the stems (27). Cowley and Bennett (46), utilizing many different types of *C. roseus* plants grown in Australia, and under the same assay conditions, found that pink flowering plants yielded 0.85 per cent of leaf alkaloids while glabrous forms of white

TABLE 3. *Crude alkaloid yield from species of Vinca.*

Species	Part[a]	Habitat	Per cent	Reference
V. minor	lf	Switzerland	0.35	77
V. minor	lf		0.86	78
V. minor	ls	Pennsylvania	0.40	79
V. minor	ls	Prague	0.50–0.90	82
V. minor	ls		0.56–1.16	36
V. minor	wp	France (Paris)	0.53	69
V. minor	wp	France (Paris)	0.34	69
V. major var. *major*	ls	Yugoslavia	0.36	94
V. major var. *major*	wp	Shores of Black Sea	0.34	87
V. major var. *major*	wp	France (Paris)	0.34	69
V. major var. *major*	wp	France (Paris)	0.54	69
V. major var. *major*	ls	France	0.035	89
V. major var. *difformis*	lf	Spain	1.10	72
V. major var. *difformis*	st	Spain	0.60	74
V. major var. *difformis*	lf	Spain	0.8–1.10	74
V. major var. *difformis*	wp	France (Var)	0.38	69
V. major var. *difformis*	wp	Corsica	0.62	69
V. herbacea var. *herbacea*	ls		0.75	69
V. herbacea var. *herbacea*	rt	Northern Caucasus	1.50	67
V. herbacea var. *libanotica*	ls	USSR	0.26	71
V. herbacea var. *libanotica*	wp	Lebanon	0.92	69
V. herbacea var. *libanotica*	rt	USSR	2.11	71
V. herbacea var. *libanotica*	rt	USSR	1.20	71
V. herbacea var. *libanotica*	rt	USSR	1.80	71

[a]*lf*, leaves; *st*, stems; *ls*, leaves and stems; *wp*, whole plant; *rt*, roots.

flowering plants contained only from 0.59 to 0.62 per cent of leaf alkaloids. Since these results were obtained by investigators using plant material collected from the same geographic area and under identical assay conditions, it would indicate that perhaps a thorough investigation of qualitative and quantitative aspects of alkaloids of the different Periwinkles should be conducted under controlled conditions.

Crystalline Alkaloids Isolated from Species of Vinca.—At least 22 crystalline alkaloids have been isolated from four of the *Vinca* species. These alkaloids, together with certain physical data have been listed in table 4. The greatest amount of investigation has been concerned with the alkaloids of *V. minor*, perhaps because of its potential value as a useful hypotensive agent (37,44).

The first crystalline alkaloid to be isolated from *V. minor* was named minorine (76). This was followed in 1953 by vincamine, isolated by Schlittler and Furlen-

meier (77). Several other investigators have reported on the isolation of vincamine from this same plant (78,80-82,84–86,124) and Janot (74) has reported its presence in *V. major* var. *difformis*. Čekan (83), in collaboration with Zabolotnaja, who originally isolated minorine, have recently demonstrated that vincamine and minorine are identical. Janot *et al.* (74) have also isolated an isomer of vincamine from *V. minor* for which they propose the name vincamirine. Perivincine, isolated from *V. minor* by Scheindlin and Rubin (79) and from *V. major* var. *major* by Farnsworth *et al.* (94), is thought by Trojánek to be a mixture of two alkaloids, vincine and vincamine (86).[2]

In 1953, Pailer and Belohlav (78) isolated an isomer of vincamine from *V. minor* and named it isovincamine. Szász *et al.* (124), Trojánek *et al.* (82-85) and Čekan *et al.* (84) have duplicated this work. Szász *et al.* (81) have shown that the vincamine-isovincamine ratio in *V. minor* total alkaloids is 4:1. An efficient method for the paper chromatographic separation of these two isomers has been developed by Debska and Kaczmarek (126) utilizing Whatman #4 paper, buffered to *p*H 4.4 and a mobile phase of peroxide-free ether. Trojánek *et al.* (85) have isolated vincaminorine and vincamidine in addition to vincine (86) from *V. minor*.

In 1934 Orechoff *et al.* isolated three crystalline alkaloids from *V. pubescens* (87,88). This species is now thought to be synonymous with *V. major* var. *major* (6). Two of the alkaloids were pubescine and vinine while a third alkaloid (mp 194–195° C), isolated only in small amounts, was unnamed. Twenty years later, Janot and LeMen (89) succeeded in isolating reserpinine (11-methoxy-δ-yohimbine) (fig. 4) from *V. major* var. *major*. A second alkaloid (mp 316° C) was also reported present in small amounts by these investigators who indicated that it resembled serpinine. Serpinine has been shown by Bose (141) and Djerassi *et al.* (95) to be identical with tetraphyllicine (fig. 4). Janot and LeMen in 1955 (90) reported on the isolation of vincamajoridine from this same plant and Janot *et al.* (142) have shown that this alkaloid is identical with akuammine (145), previously isolated from the seeds of *Picralima nitida* and *P. klaineana* (143,144) (fig. 4). The fourth alkaloid isolated from *V. major* var. *major* by Janot and LeMen (91) was vincamajoreine, present only in minute amounts. These same workers (92) have also isolated vincamajine from this plant while Goodwin and Horning (93) have reported its presence in *Tonduzia longifolia*. The relationship of the structure of vincamajine to vincamedine has been studied by Janot *et al.* (146) and these workers have shown that vincamedine is O-acetylvincamajine. The most recent alkaloid isolated from *V. major* var. *major* is perivincine (94), previously isolated only from *V. minor* by Scheindlin and Rubin (79).

V. major var. *difformis* has been investigated by Janot *et al.* (72,74) who have isolated vincamedine (72), vincamine and sarpagine (74) (fig. 4). The structure of sarpagine has been studied by Poisson *et al.* (73) and found to be O-methyl lochnerine. Lochnerine has not been reported as being present in any of the *Vinca* species but has been isolated from *C. roseus* (102,103,110) (fig. 4).

Crystalline Alkaloids Isolated from Species of Catharanthus.—Vincain (99) and vinceine (100) were the first alkaloids to be isolated from *C. roseus*. In 1955 Chatterjee and Talapatra (101) prepared a purified form of vincain and reported that it was identical or stereoisomeric with δ-yohimbine (ajmalicine) (fig. 4). This was confirmed by Mors *et al.* (102) in 1956, who also indicated that perhaps vincain was identical with ajmalicine. These workers were able to isolate both ajmalicine and serpentine from the whole plants of *C. roseus* f. *albus* in addition to a new alkaloid for which they proposed the name lochnerine. In 1956, Janot and LeMen (103) also isolated ajmalicine and lochnerine from *C. roseus* in addi-

[2]The sample of perivincine used by Trojánek *et al.* in determining this was supplied by this author. It was chromatographically pure when isolated (94). The small amount on hand did not permit recrystallization prior to sending it to Dr. Trojánek and it probably had decomposed to some degree. Experiments are now in progress to investigate this possibility.

tion to akuammine (vincamajoridine). Ajmalicine has also been isolated from *C. roseus* by El-Deeb *et al.* (104), Pillay *et al.* (106), Shimizu and Uchimaru (108), Svoboda *et al.* (117), Inagaki *et al.* (119) and Kohlmunzer and Krupinska (120). Tetrahydroserpentine (ajmalicine monohydrate) has been reported from this same species by Svoboda *et al.* (110,117) while serpentine, originally isolated from

TABLE 4. *Alkaloids isolated from Vinca species.*

Alkaloid	Formula	mp C	Plant[a] part	Species[b]	pK'_a[c]	$[\alpha]_D$	Temp C	Solvent	Reference
Akuammine	See vincamajoridine								
Isovincamine	$C_{21}H_{26}N_2O_3$	218–219°	lf	A	—	+28°±2°	15°	Pyridine	78,81,82
	$C_{21}H_{26}N_2O_3$	218–220°	ls	A	—	+26°±2°	23°	Pyridine	
Minorine	See vincamine								
Perivincine	$C_{23}H_{26}N_2O_4$	201–202° dec	ls	A	—	—	—	—	79,94
	$C_{22}H_{28}N_2O_4$	199.5–200° dec	ls	B	8.20	−61°±2°	27°	Pyridine	
Pubescine	$C_{20}H_{26}N_2O_4$	227–228°	wp	B	—	−134.2°	—	Abs EtOH	87,88
Reserpinine	$C_{22}H_{26}N_2O_4$	242°	ls	B	—	−109°±4°	—	Pyridine	70,75,89
	$C_{22}H_{26}N_2O_4$	243–244° dec	—	—	—	−131°	26°	$CHCl_3$	
Sarpagine	$C_{19}H_{22}N_2O_2$	310° dec	—	—	—	+53.4°	24°	Pyridine	73-75
	$C_{19}H_{22}N_2O_2$	>360°	st	C	—	+55°±2°	20°	Pyridine	
	$C_{19}H_{22}N_2O_2$	No data given	—	C	—	—	—	—	
Serpinine	See tetraphyllicine								
Tetraphyllicine	$C_{20}H_{24}N_2O$	320–322°	—	—	—	+21°	27°	Pyridine	89,95
—	—	316°	ls	B	—	—	—	—	
Vincamajine	$C_{22}H_{26}N_2O_3$	225°	ls	B	—	−55°±5°	—	EtOH	92,93
	$C_{22}H_{26}N_2O_3$	225–226°	—	—	—	−48°	25°	Abs EtOH	
Vincamajoreine	$C_{21}H_{26-28}N_2O_2$	246–247° (cap) 271° (block)	ls	B	—	—	—	—	91
Vincamajoridine	$C_{22}H_{26}N_2O_4$	252° (vac cap)	ls	B	—	−104°±4°	—	Pyridine	75,90
	$C_{22}H_{26}N_2O_4$	258–260° dec	—	—	—	−104°	—	Pyridine	
Vincamedine	$C_{24}H_{26-28}N_2O_4$	185°	ls	C	—	−66°±2°	20°	$CHCl_3$	72,74
	$C_{24}H_{28}N_2O_4$	—	lf	C	—	—	—	—	
Vincamidine	$C_{20}H_{24}N_2O_3$	78–80°	lf	A	—	—	—	—	84,85
Vincamine	$C_{21}H_{26}N_2O_3$	232–233°	lf	A	—	+41°±4°	23°	Pyridine	34,71,74
	$C_{21}H_{26}N_2O_3$	231–232°	lf	A	—	+39°±2°	15°	—	76-78,80-82
	$C_{21}H_{26}N_2O_3$	232–233.5°	ls	A	—	+40°±2°	23°	Pyridine	
	$C_{21}H_{26}N_2O_3$	230–231°	ls	A	—	—	—	—	
	$C_{21}H_{26}N_2O_3$	235–237°	—	A	—	+38°±2°	20°	Pyridine	
	$C_{21}H_{26}N_2O_3$	258° (block) 222° (cap cor)	st	C	—	+43°	—	Pyridine	
	$C_{22}H_{26}N_2O_2$[d]	223–224°	ls	A	—	—	—	—	
	$C_{22}H_{26}N_2O_3$[d]	224–225°	ls	D	—	—	—	—	
Vincaminoreine	$C_{22}H_{30}N_2O_2$	126°	ls	A	—	—	—	—	84,85
		124–126°	ls	A	—	—	—	—	
Vincaminorine	$C_{22}H_{30}N_2O_2$	130–131°	ls	A	—	+46°±2°	21°	EtOH	82,84
Vincamirine	$C_{21}H_{26}N_2O_3$	271° (block)	—	C	—	−62°±4°	—	$CHCl_3$	74
		231° (cap)	—	C	—	+33°±4°	—	Pyridine	
Vincanidine	$C_{20}H_{24}N_2O_3$	Chars at 250–280°	wp	D	—	—	—	—	70,71
Vincanine	$C_{19}H_{22}N_2O$	187.5–188°	rt	D	—	−992°	17°	CH_3OH	71
Vincine	$C_{22}H_{28}N_2O_4$	212–214°	ls	A	—	+36±2°	20°	Pyridine	86
Vinine	$C_{19}H_{26}N_2O_4$	211.5–213°	wp	B	—	−70.12°	—	Abs EtOH	87,88
Alkaloid	—	194–195°	wp	B	—	—	—	—	87,88
Alkaloid	—	124–126°	ls	A	—	—	—	—	82

[a] *lf*, leaves; *ls*, leaves and stems; *wp*, whole plant; *rt*, roots; *st*, stems.
[b] *A*, *V. minor*; *B*, *V. major* var. *major*; *C*, *V. major* var. *diformis*; *D*, *V. herbacea* var. *libanotica*.
[c] In 75 per cent DMF.
[d] Originally named minorine but shown to be identical with vincamine (83).

TABLE 5. *Alkaloids isolated from Catharanthus species.*

Alkaloid	Formula	mp C	Plant part[a]	Species[b]	pK'_a[c]	$[\alpha]D$	Temp C	Solvent	Reference
Ajmalicine	$C_{21}H_{24}N_2O_3$	253–255°	rt	C	—	−54°	23°	Pyridine	96,99-104
		254–256°	rt	C	—	−58°	19°	Pyridine	106,108,117
		259–260°	rb	C	—	—	—	—	119,120
		255–257°	wp	C	—	—	—	—	
		No data given	wp	C	—	—	—	—	
		No data given	wp	D	—	—	—	—	
Akuammine	See Vincamajoridine								
Catharanthine	$C_{21}H_{24}N_2O_2$	126–128°	wp	C	6.8	+29.8°	26°	$CHCl_3$	114,117
Catharine	$C_{46}H_{52}N_4O$	271–275° dec	wp	C	5.34	−54.2°	26°	$CHCl_3$	121
Isoleurosine	$C_{46}H_{60}N_4O_9$	202–206° dec	wp	C	4.8,7.3	+61.2°	26°	$CHCl_3$	121
Lanceine	$C_{20}H_{26}N_2O_3$ or $C_{24}H_{30}N_2O_4$	198°	rt	D	—	+64±4°	20°	EtOH	97
						+62±4°	20°	$CHCl_3$	
Leurosine	$C_{46}H_{58}N_4O_9$	202–205° dec	wp	C	5.5,7.5[d]	+72	26°	$CHCl_3$	110,115-117
		200–203° dec	wp	C	—	+59.8	25°	$CHCl_3$	
		202–205° dec	wp	C	5.5,7.5[d]	+72°	26°	$CHCl_3$	
Lochnericine	$C_{21}H_{24}N_2O_3$	190–193° dec	wp	C	4.2	−432°	27°	$CHCl_3$	114,117
	$C_{21}H_{24-26}N_2O_3$	188–189°	rb	C	—	−36.1°	16°	EtOH	118
Lochneridine	$C_{19}H_{24}N_2O_3$	211–214° dec	wp	C	5.5	—	—	—	121
Lochnerine	$C_{20}H_{24}N_2O_3$	202–203°	wp	C	—	+52°	24°	Pyridine	102,103
		200–201°	rb	C	—	+66.83°	28°	EtOAc	110
	$C_{20-1}H_{26-8}N_2O_2$	200–201°	wp	C	—	+50°	21°	EtOH	
		No data given	wp	C	—	—	—	—	
		No data given	rt	C	—	—	—	—	
Perivine	$C_{20}H_{22}N_2O_3$	218–221° dec	wp	C	7.5	−121.4°	26°	$CHCl_3$	110,117
Reserpine	$C_{33}H_{40}N_2O_9$	264–265° dec			—	−115°	26°	$CHCl_3$	107,111,112
		265–266° dec	rt	C	—	—	—	—	
		262–263° dec	rt	C	—	—	—	—	
Serpentine	$C_{21}H_{20}N_2O_3$	190–200° dec (nitrate)	rt	C	—	—	—	—	102,105,106
		160–161° (HBr) and 260–261° (HBr)	rb	C	—	—	—	—	108,114,117
		265–267° (HBr)	rb	C	—	—	—	—	
		157–158°	rb	C	—	—	—	—	
		156° dec	wp	C	—	—	—	—	
		No data given	wp	C	—	—	—	—	
Sitsirikine	$C_{21}H_{26}N_2O_3$	239–241° dec (½ H_2SO_4)	wp	C	7.6	+23° (base)	26°	$CHCl_3$	121
Tetrahydroalstonine	$C_{21}H_{24}N_2O_3$	225°	wp	C	—	−86°	21°	Pyridine	108,110,117
	$C_{21}H_{24}N_2O_3$	230–231° dec			—	−102.3°	26°	$CHCl_3$	
		No data given	wp	C	—	—	—	—	
	$C_{21}H_{24}N_2O_3$	231°	rt	D	—	−96°±4°	20°	Pyridine	98
Tetrahydroserpentine (Ajmalicine monohydrate)	$C_{21}H_{24}N_2O_3 \cdot H_2O$	253–255° dec	—	—	—	−39°	26°	Pyridine	110
		No data given	wp	C	—	—	—	—	
Vincain	See Ajmalicine								
Vincaleukoblastine	$C_{46}H_{58}N_4O_9$	211–216° dec (etherate)	wp	C	5.4,7.4 (etherate)	+42° (etherate)	26°	$CHCl_3$	1,114-117
	$C_{46}H_{58}N_4O_9$	201–211° (etherate)	wp	C	—	+42° (etherate)	26°	$CHCl_3$	
	$C_{24}H_{35}N_2O_7$[e]	192–195° No data given	lf	C	—	−32.2°	23°	CH_3OH	
Vincamajoridine	$C_{22}H_{26}N_2O_4$	252° (vac)	rt	C	—	−104°±4°	—	Pyridine	103
Vincamicine		224–228° dec	wp	C	4.80,5.85	+418.0°	26°	$CHCl_3$	121
Vinceine	See Ajmalicine		rt	C	—	—	—	—	
Vindolicine	$C_{25}H_{32}N_2O_6$	248–251° –melts recryst. then 265–267° dec	wp	C	—	—	—	—	121
					5.4	−48.4°	26°	$CHCl_3$	
Vindoline	$C_{27}H_{34}N_2O_6$[e] .½ H_2O	150–152°	lf	C	—	—	—	—	109,114,117
	$C_{25}H_{32}N_2O_6$	154–155°	wp	C	5.5	−18°(?)	26°	$CHCl_3$	
	$C_{25}H_{32}N_2O_6$	154–155°	wp	C	5.5	+42°	27°	$CHCl_3$	
Vindolinine 2 HCl	$C_{21}H_{24-26}N_2O_2$ .2 HCl	210–212° dec	wp	C	3.3,7.1	−8°	26°	H_2O	113,117
	$C_{21}H_{24}N_2O_2$ .2 HCl	250–252° (block)	rt	C	—	−26.8°	20°	H_2O	
Virosine	$C_{22}H_{26}N_2O_4$	257–262° dec	wp	C	—	—	—	—	
		258–261° dec No data given	wp	C	5.85	−160.5°	26°	$CHCl_3$	110,117
Yohimbine (Quebrachine)	$C_{21}H_{26}N_2O_3$	246° (block)	rt	D	—	+55°±1°	18°	EtOH	97
		245° (cap)	—	—	—	+11°±42°	18°	$CHCl_3$	
δ-yohimbine	See Ajmalicine								
Alkaloid		183°	rt	D	—	—	—	—	97
Alkaloid		220°	—	C	—	—	—	—	104
Alkaloid		255–259°	—	C	—	—	—	—	109
Alkaloid		242°	—	C	—	—	—	—	107

[a] *rt*, roots; *st*, stems; *lf*, leaves; *ls*, leaves and stems; *wp*, whole plant; [b] *C*, *C. roseus*; *D*, *C. lanceus*.
[c] In 66% DMF. [d] In water. [e] Proposed formula was incorrect (115).

Rauvolfia serpentina, has been found in *C. roseus* by a number of investigators (105,106,108,114,117). Shimizu and Uchimaru (108), Svoboda (110) and Svoboda *et al.* (117) have isolated tetrahydroalstonine while Basu and Sarker (107), Nazir and Handa (111) and Bose *et al.* (112) have all reported on the isolation of reserpine from this same plant. Although Svoboda *et al.* (110,114–117) have isolated the new alkaloids leurosine, isoleurosine, virosine, perivine, catharanthine, catharine, lochnericine, lochneridine, vindoline, vindolinine, vincamicine and sitsirikine from *C. roseus* in addition to confirming the presence of ajmalicine, tetrahydroalstonine, tetrahydroserpentine, serpentine, lochnerine and vincaleukoblastine, they have not found reserpine in their studies. Similarly, this group has not found akuammine (vincamajoridine) in *C. roseus* although it was reported isolated from this plant by Janot and LeMen (103). Akuammine is the only alkaloid isolated to date from the Periwinkles that is common to both the genus *Catharanthus* and *Vinca.*

The alkaloid vindoline, reported by Kamat *et al.* (109) from *C. roseus,* and postulated as a $C_{27}H_{34}N_2O_6 \cdot 1/2H_2O$ base has been shown by Gorman *et al.* (114) to be a fused five-ring dihydroindole alkaloid with an empirical formula of $C_{25}H_{32}N_2O_6$. In addition, the carcinostatic alkaloid vincaleukoblastine which was first isolated by Noble *et al.* as a $C_{24}H_{35}N_2O_7$ alkaloid has now been demonstrated on the basis of electrometric titrations, carbon-oxygen ratios and functional group analyses (115,116), to be a $C_{46}H_{58}N_4O_9$ dimeric alkaloid containing both indole and dihyroindole moieties. Vincaleukoblastine and its isomer leurosine both appear to contain the essential structural features of catharanthine and vindoline and Gorman *et al.* (116) postulate that catharanthine and vindoline are linked in a manner which leaves the indole-NH free in the formation of vincaleukoblastine (or leurosine) by the plant. This then poses the question as to whether vindoline and catharanthine are precursors of vincaleukoblastine (or leurosine) in the plant or artifacts formed during the processing of plant material.

Other investigators isolating alkaloids from *C. roseus* include Nair and Pillay (118) who isolated lochnericine, and Janot *et al.* (113) who have demonstrated the presence of vindolinine. Basu and Sarker (107) have isolated an alkaloid (mp 242° C) which was not identified further as have Nair and Pillay (118) (mp 188–189° C) and Kamat *et al.* (109) (mp 255–259° C).

Physical data are listed for the crystalline alkaloids isolated from species of *Catharanthus* in table 5. Infrared and ultraviolet spectra as well as x-ray crystallographic data for most of the *Vinca* and *Catharanthus* alkaloids is reported by Svoboda *et al.* (117,121), Gorman *et al.* (114,116), Neuss (75), Neuss *et al.* (115) and van Camp (147).

The only other species of *Catharanthus* from which crystalline alkaloids have been isolated is *C. lanceus.* Janot *et al.* have been the only investigators to report on these alkaloids. They have isolated ajmalicine (96), yohimbine (quebrachine) and an unknown alkaloid mp 183° C (97), tetrahydroalstonine and a new alkaloid, lanceine (98). Utilizing two-dimension thin-layer chromatography in our laboratories we have demonstrated the presence of some 40 alkaloid components in this plant, about ten of which appear to be major alkaloids.

Several references have been found in the literature concerning the isolation of an alkaloid named vincarosine from *C. pusillus.* Chopra *et al.* (153) in their book *Indigenous Drugs of India* have cited the following under the monograph for *Lochnera pusilla* (*C. pusillus*): "It is a cardiac poison and *Lochnera pusilla* is regarded as poisonous to cattle. They contain an amorphous alkaloid vincarosline." The reference cited for this was Boorsma (152). Boorsma, in his publication investigated *C. roseus* f. *albus* and did not mention vincarosline or vincarosine; however, he cited the work of Greshoff (155) who investigated *C. roseus* also and there was no mention of specific alkaloids in this publication. Perhaps the confusion has arisen from the fact that Chopra *et al.* (153) have considered *C. roseus* as a synonym for *C. pusillus.* In the work *Poisonous Plants of India,* also by

Chopra *et al.* (49), separate monographs are recognized for *C. roseus* and for *C. pusillus*. Under the *C. pusillus* monograph, these authors mention the following: "Very little is known regarding the medicinal properties of this plant. It contains an amorphous alkaloid named vincarosine, which is a heart poison." The references cited for the vincarosine isolation were Boorsma (152) and Greshoff (155) who, as previously mentioned, used *C. roseus* in their studies and did not mention

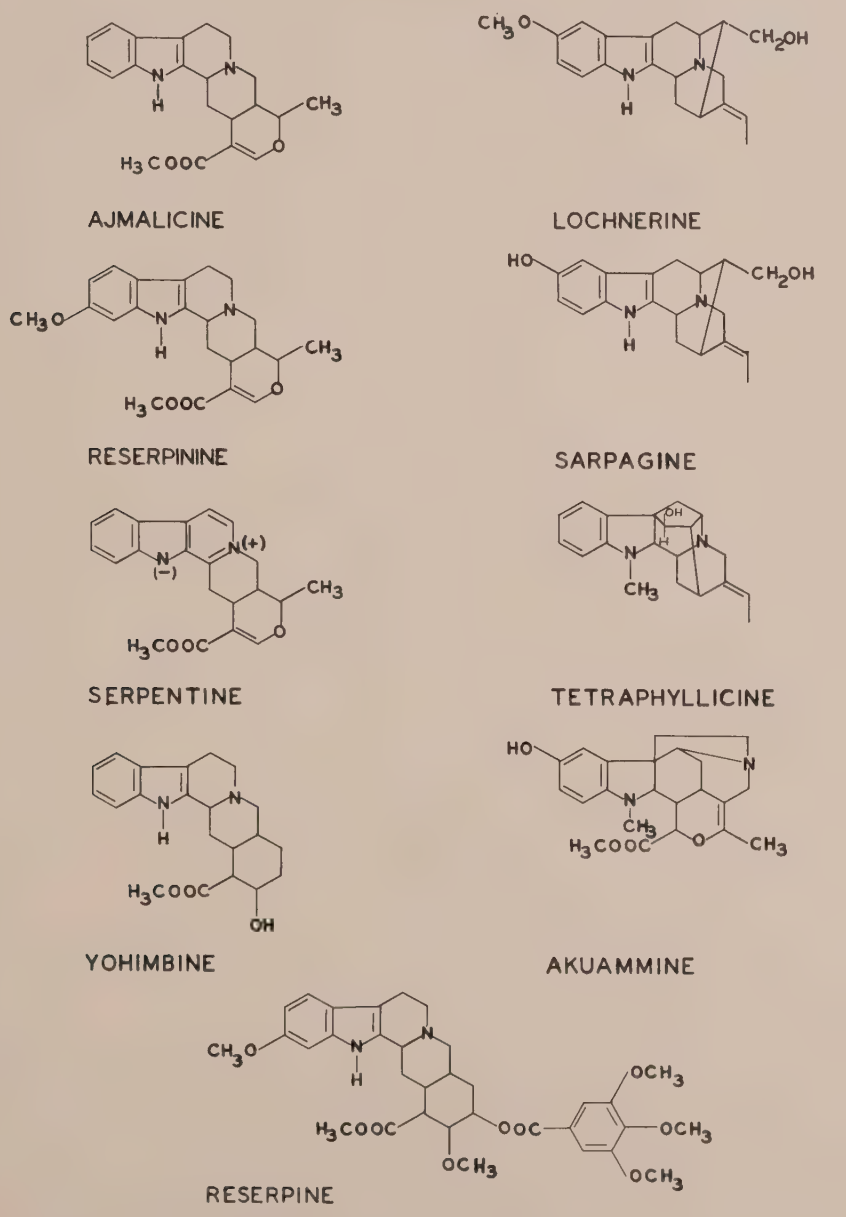

FIG. 4. *Alkaloids of known structure from Vinca and Catharanthus species.*

vincarosine. Perhaps some of this confusion is centered around the publication of Dymock *et al.* (156) who report that in their investigation of *C. pusillus*: "We have chemically investigated this plant, and find that the poisonous property is due to an alkaloid vincine, which is distinguished by giving a carmine-red colour with pure nitric acid."

In any event, no original reference has been found which indicates the origin of the alkaloid vincarosine.

Non-alkaloid constituents of the Periwinkles.—Rutishauser (39,40) has noted the presence of several tannoids in the leaves of *V. minor*, one of which, a glucoside, was given the name "vincoside" while a second was thought to be in combination with pyrocatechuic acid. King *et al.* (34) have shown that this "vincoside" is 3-β-*D*-Glucosyloxy-2-hydroxybenzoic acid. Bridel and Charaux

TABLE 6. *Non-alkaloid constituents of Vinca species.*

Constituent	Species	Remarks	Reference
Caffeic acid	*V. minor*	Detected in acid hydrolysates	133
Choline		Doubtful presence by paper chromatography	131
p-coumaric acid		Detected in acid hydrolysates	133
Dambonitol		Isolated	132
Fructose		Isolated	34
Gentisic acid		Detected in acid hydrolysates	133
3-β-*D*-Glucosyloxy-2-hydroxy benzoic acid		Isolated	34
p-hydroxybenzoic acid		Detected in acid hydrolysates	133
Ornol		Isolated	35
Protocatechuic acid		Detected in acid hydrolysates	133
o-protocatechuic acid		Detected in acid hydrolysates	133
Robinoside		Isolated	127
Rubber		Isolated	36,130
β-sitosterol		Isolated	35
Sucrose		Isolated	34
Tannins		Detected	34,39,40
Triacontane		Isolated	35
Ursolic acid		Isolated	32–36,82
Vanillic acid		Detected in acid hydrolysates	133
Vincosides		Detected	39,40,66
Carotene	*V. major* var. *major*	Detected	134
Dambonitol		Isolated	132
Glucosides		Detected	140
o-protocatechuic acid		Detected in acid hydrolysates	128
Robinoside		Isolated	127
Ursolic acid		Isolated	33,184
β-glucosidase	*V. herbacea* var. *herbacea*	Detected	66
Saccharase		Detected	66

have also detected glucosides hydrolyzed by rhamnodiastase in *V. major* var. *major* (140). Ibrahim and Towers (133) have detected *p*-coumaric, *p*-hydroxybenzoic, *o*-pyrocatechuic, gentisic, protocatechuic, vanillic, and caffeic acids in acid hydrolysates of *V. minor* leaves as well as *o*-pyrocatechuic acid in similar acid hydrolysates of *V. major* var. *major* and *C. roseus* (128). Related anthocyanidins have also been isolated from *C. roseus* acid hydrolysates by Forsyth and Simmonds (139). Petunidin and malvidin were found as minor components and hirsutidin (tentative) was described as the major component derived from glycosides in the plant. Three anthoxanthins were also found in the flowers by the same workers, one being kaempferol and a second was thought to be quercetin.

Hajkova *et al.* (36) and Mitchell (130) have reported on the isolation of rubber from *V. minor* leaves in from 1.0 to 2.0 per cent yields. The triterpenoid saponin ursolic acid appears to be widespread in the Periwinkles, having been found in *V. minor* by LeMen and Pourrat (32,184), King *et al.* (34), LeMen and Hammouda (35), Hájková *et al.* (36) and Trojánek *et al.* (82) while LeMen and Pourrat (184) have isolated it from *V. major* var. *major* and Roy and Chatterjee (135) from *C. roseus*.

It is difficult to interpret the screening results of Wall *et al.* (136) with respect to saponin testing when it is known that ursolic acid, a hemolytic triterpenoid compound, has been isolated from both *V. minor* and *C. roseus*. Both of these plants were reported by these workers as giving negative hemolytic saponin tests (137,138). In addition, Roberg (129) also has indicated that *V. minor* contains no saponins.

Wall *et al.* (137,138) have also screened *V. minor*, *V. major* var. *major* and *C. roseus* for tannins with negative results for all three plants while Aldaba *et al.*

TABLE 7. *Non-alkaloid constituents of Catharanthus species.*

Constituent	Species	Remarks	Reference
Anthoxanthins	*C. roseus*	Isolated	139
d-camphor		Isolated	106
Choline		Detection by chromatography	131
Formic acid		Isolated	31
Hirsutidin		Detection in acid hydrolysates	139
Kaempferol		Isolated	139
Lochnerallol		Isolated	31
Lochnerol		Isolated	31
Malvidin		Detection in acid hydrolysates	139
Mannoside		Detected	31
Neutral principle		Isolated	106
Palmitic acid		Isolated	31
Petunidin		Detection in acid hydrolysates	139
o-pyrocatechuic acid		Detection in acid hydrolysates	128
Quercetin		Isolated	139
β-sitosterol		Isolated	117
Stearic acid		Isolated	31
Tannins		Detected	31
Ursolic acid		Isolated	135
Volatile oil		Isolated	31
Choline	*C. lanceus*	Detection by chromatography	131
Tannins		Detected	48

(31) have shown evidence of tannins in *C. roseus* and Rutishauser (39,40) and King *et al.* (34) have reported the presence of tannins in *V. minor*.

Other non-alkaloid constituents isolated from or detected in *V. major* var. *major* are dambonitol (132) and carotene (134). Also ornol, β-sitosterol and triacontane (35) as well as dambonitol (132) were isolated from *V. minor* leaves. Paris and Moyse-Mignon (131) have detected choline in *C. lanceus* plants by paper chromatography while tannins were reported in plants growing in Africa (48). Aldaba *et al.* (31) have isolated formic, stearic and palmitic acids, lochnerol, lochnerallol, a mannoside and a volatile oil in addition to detecting tannins in the air-dried leaves of *C. roseus* plants. Choline has also been detected in the roots of this plant by paper chromatography (131) and an unidentified neutral principle (mp 148° C) has also been isolated from *C. roseus* (106). See tables 6 and 7.

CHEMOTAXONOMY

Knowledge of the chemistry of the Periwinkles at the present time is quite incomplete and a number of species remain to be investigated. A number of chemical compounds have been isolated from certain species by a host of investigators and a number of other substances have been qualitatively detected. The investigations, for the greatest part have been on single species with only a few researchers making any attempt to compare the constituents of several species at

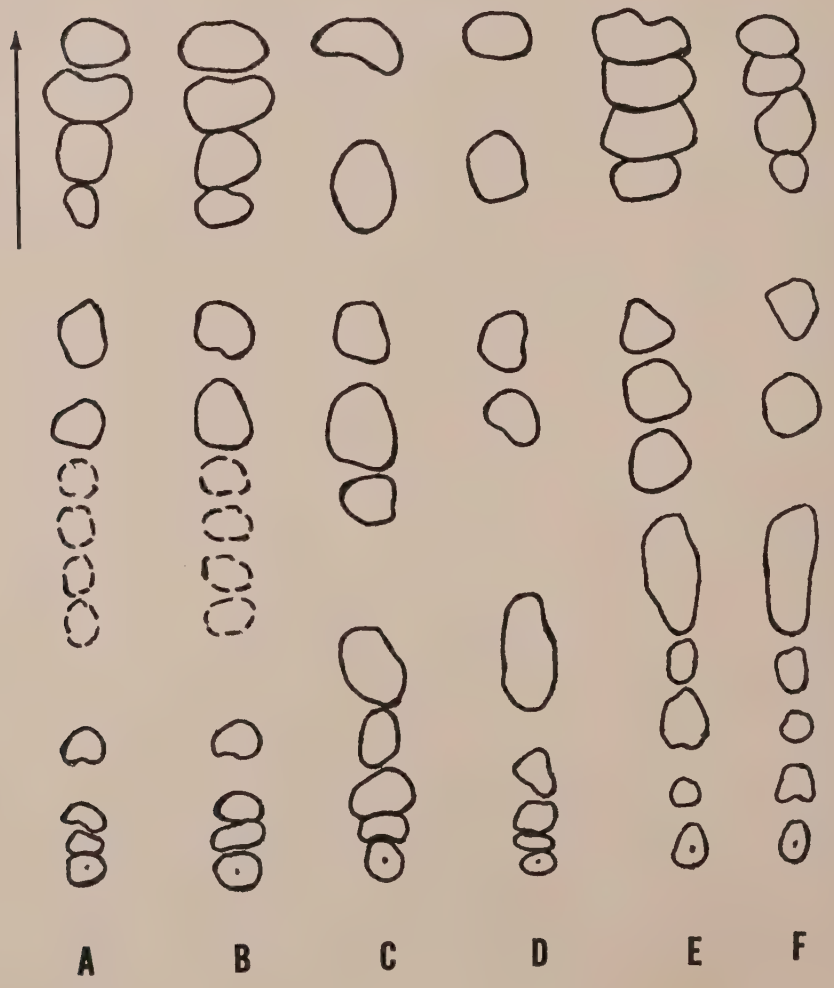

FIG. 5. *Thin-layer chromatogram of Vinca and Catharanthus alkaloids. A, C. roseus (pink flowering); B, C. roseus f. albus; C, V. major var. major; D, V. minor; E, C. lanceus, whole plant; F, C. lanceus, leaves and stems. Solid outlines indicate a positive Dragendorff reaction. Broken outlines indicate fluorescent spots which were Dragendorff negative.*

the same time. Perhaps one of the reasons for this is the apparent inability to obtain sufficient plant material for investigation. *C. scitulus* and *C. trichophyllus* are indigenous to Madagascar, but are only found scattered, and it may be that not enough of these plants are available for collection. In the case of *C. pusillus*,

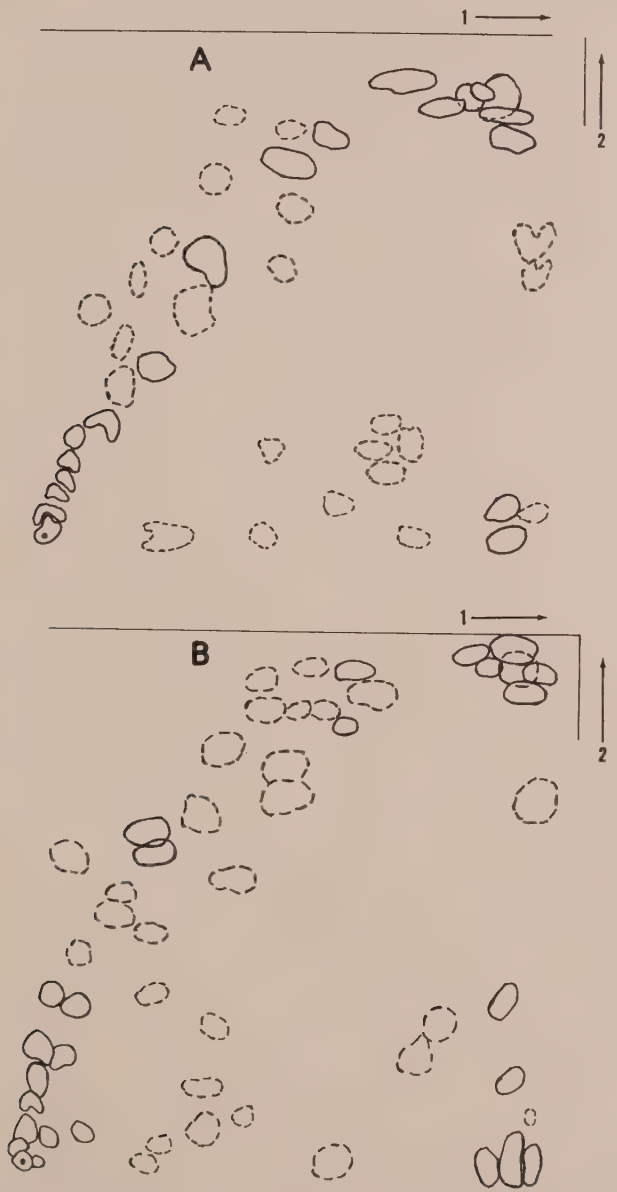

FIG. 6. *Two-dimensional thin-layer chromatograms of Catharanthus alkaloids.*
A. Catharanthus lanceus total alkaloids.
B. Catharanthus roseus (pink flowering) total alkaloids.
Solid outlines indicate a positive Dragendorff reaction. Broken outlines indicate fluorescent spots which were Dragendorff negative.

which from literature references appears to be widespread in India, it is difficult to understand the apparent lack of investigation. We have been able to obtain seeds so named from India but found that mature plants produced from them were *C. roseus*.

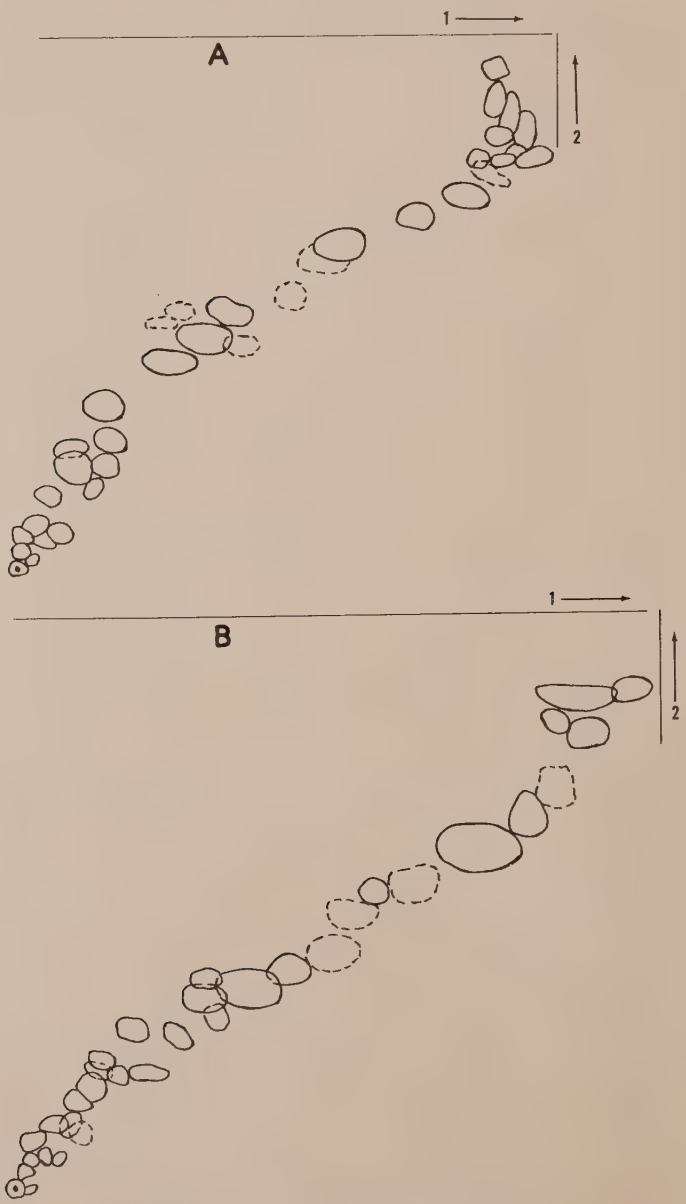

FIG. 7. *Two-dimensional thin-layer chromatograms of Vinca alkaloids.*
A. Vinca minor total alkaloids.
B. Vinca major var. major total alkaloids.
Solid outlines indicate a positive Dragendorff reaction. Broken outlines indicate fluorescent spots which were Dragendorff negative.

Paris and Moyse (69) have compared the paper electrophoretic alkaloid patterns of *C. roseus*, *C. lanceus*, *C. longifolius*, *C. trichophyllus*, *V. minor*, *V. major* var. *major*, *V. major* var. *difformis*, *V. herbacea* var. *herbacea* and *V. herbacea* var. *libanotica*. They concluded that the alkaloid electrophoretic patterns of the *Catharanthus* species investigated were of sufficient clarity as to distinguish them from the *Vinca* species as classified by Pichon (5,6). This is the first reported chemical evidence substantiating the botanical taxonomy of *Vinca* and *Catharanthus* species.

The following data also illustrates the differences in alkaloid distribution within these two groups of plants. In a study conducted by the author, alkaloids were isolated from *V. minor*[3] leaves and stems collected in New Jersey during June, 1960; *V. major* var. *major* leaves and stems collected during the summer of 1957 in Yugoslavia;[3] *C. lanceus* whole plants collected in Madagascar during 1960[3] and *C. roseus* (pink flowering) whole plants grown from seed in Pittsburgh, Pa. in 1960.

The alkaloids were extracted from each of these species in the following manner. The dried powdered plant material was thoroughly wetted with 28 per cent ammonium hydroxide solution and allowed to stand overnight and then extracted continuously with chloroform. The chloroform solution was dried over anhydrous sodium sulfate, filtered and the filtrate evaporated to dryness *in vacuo*. The residue was heated with 6 per cent acetic acid solution for one hour with continuous stirring, filtered, and the residue washed with acetic acid solution until a negative test for alkaloids was obtained. The acid solution of alkaloids was treated with 28 per cent ammonium hydroxide solution to *p*H 9.5 and the alkaloids removed by extraction with chloroform. The chloroform solution was dried over anhydrous sodium sulfate, concentrated *in vacuo* to a small volume, and evaporated to dryness in a current of air.

Five per cent solutions of these alkaloids in chloroform were prepared for use in thin-layer chromatography. Silica Gel G and the DeSaga apparatus[4] were used with 200 × 200 mm glass plates.

One Dimensional Thin-Layer Chromatography.—A developing solvent of chloroform/methanol (95:5) provided maximum separation. The silica gel plates were prepared in the usual manner and heated at 100° C for 30 minutes. After the plates had cooled to room temperature, 5 μl of each sample was applied. The plates were developed in air-tight glass chambers in about 1 hour. The plates were removed, air-dried, and each fluorescent spot (long-wave ultraviolet light) was outlined. The plates were sprayed with Dragendorff's reagent. The results are shown in figure 5. It can be seen by the relative number and position of the separated spots, that a clear-cut difference exists between the two species of *Catharanthus* and the two species of *Vinca*.

Two Dimensional Thin-Layer Chromatography.—Plates were prepared in the same manner as described above. Single applications of each of the four samples of alkaloids were placed on separate plates and the plates were each developed with chloroform/methanol (95:5) in the first direction and chloroform/methanol (90:10) for the second direction. Although the fluorescent colors on the completed chromatograms are not noted in the illustrations (fig. 6 and 7), the characteristic separation pattern clearly points out the differences between the alkaloids of the two *Vinca* and the two *Catharanthus* species.

BIOLOGICAL PROPERTIES OF THE PERIWINKLES

General Pharmacology.—Comprehensive reviews concerning the pharmacology of several alkaloid-containing preparations in addition to purified alkaloids pre-

[3]Supplied by the S. B. Penick Company, 100 Church Street, New York 8, New York.

[4]DeSaga apparatus for thin-layer chromatography and Silica Gel G. Brinkmann Instruments Inc., Great Neck, L.I., New York.

pared from *C. roseus*, *V. minor* and *V. major* var. *major* have been published (11, 14,157,158). The purpose of this section is not to critically review the pharmacology of the Periwinkles and their constituents, but rather to provide the interested investigator with a summary of those reports of biological activity associated with these plants.

The biological activity of *V. minor* extracts in several species of animals has been reported by Hano and Maj (159,160) and the general pharmacologic properties of the total alkaloids from this plant have been evaluated by Quevauviller *et al.* (161,162). In addition, the action of galenical preparations of *V. minor* have been noted on the perfused guinea pig heart (163). Szczecklik *et al.* (37) treated several patients suffering from arterial hypertension with powdered *V. minor* herb in oral doses of 3 g daily and concluded that the results obtained were equivalent to those expected in patients treated for similar conditions with reserpine, barbiturates or purines. In animal studies, Zheliazkov (164) has shown that *V. minor* total alkaloids not only produced a marked hypotensive activity, but also a curare-like action. This investigator expressed the opinion that possibly the total alkaloids are composed of at least two general types, the stronger type eliciting the hypotensive activity and a weaker type carrying the curare-like action. Sultanov (165) later reported on the occurrence of strychnine-like convulsions in rabbits that had received 7 mg/kg of vincamine hydrochloride.

Vincamine is thought to be the chief hypotensive alkaloid of *V. minor*. Its pharmacologic properties have been studied by Raymond-Hamet (43), Szporny and Szász (42), Čekan *et al.* (84), and Krejči (41). Szabó and Nagy (44) have shown that a purified alkaloid mixture from *V. minor* (Devincan) induces a marked hypotensive response in animals and favorable responses were noted in 16 of 31 hypertensive patients treated with daily oral doses of from 5 to 20 mg. The most favorable results were observed in those patients suffering with essential hypertension.

Vinca major var. *major* total alkaloids have been reported by Orechoff *et al.* (87), to have marked hypotensive properties. These investigators have also pointed out that one of the three alkaloids isolated in their study, vinine, elicited hypotensive activity. Quevauviller *et al.* (166) have reported on the pharmacologic properties of *V. major* var. *major* total alkaloids in dogs and found that doses of 10 mg/kg (iv) produced a marked fall in blood pressure. Two other alkaloids isolated from this plant, reserpinine (157) and perivincine (94), have been shown to exhibit only transient hypotensive effects.

The general pharmacology of the crude alkaloids from *C. roseus* has been reported by Sheth *et al.* (123), Neogi and Bhatia (125), Abu-Shady *et al.* (167), Chopra *et al.* (151) and Paris and Moyse (100). Either the total aklaloids or fractions obtained from the total alkaloids were reported by these investigators to have significant hypotensive activity in anesthetized dogs.

Anti-diabetic Activity of Catharanthus roseus.—The wide use of this plant as an oral anti-diabetic remedy has been previously discussed (45, 47, 50–55). Noble *et al.* (1), Epstein (51), Lee and Drew (52), and Corkill and Doutch (55) have obtained essentially negative results with *C. roseus* extracts in normal, as well as in experimentally induced hyperglycemic rabbits. Garcia (53), in a study utilizing only three rabbits, has shown that fresh dried, one-week old and one month old dried leaves of *C. roseus* in the form of decoctions, equivalent to 2 g of drug, induced drops in normal blood sugar levels ranging from about 10 to 25 per cent. Corkill and Doutch (55) and Nye and Fitzgerald (54) obtained essentially negative results in clinical studies with diabetic patients. A recent report by Repiton and Guillaumin (168) has shown that they were able to lower both normal as well as high blood sugar levels by using *C. roseus* tincture or decoction. This report prompted Paris and Moyse (14) to re-investigate the anti-diabetic properties of this plant. They used normal rats and rabbits, alloxan-induced hyper-

glycemic rats and oral-glucose-induced hyperglycemic rabbits in this study. It was determined that subcutaneous injections of *C. roseus* extracts in normal animals produced about a 10 per cent drop in blood sugar levels after 4 days. Oral doses of the extract in alloxan-induced hyperglycemic rats produced no significant changes

TABLE 8. *Antibacterial activity of C. roseus alkaloid fractions.*[a]

Test organism	Vindoline	Alkaloid fractions		
		Fraction I	Fraction II	Fraction III
M. pyogenes var. *aureus* (FDA 209)	+[b]	+	+	++
M. pyogenes var. *albus* (MRC 1001)	−	+	+	++
M. citreus (MRC 1005)	++	++	++	++
M. epidermidis (MRC 1004)	−	+	+	++
D. pneumoniae type II (NCTC 7466)	+	+	+	++
Str. hemolyticus (ATCC 8668)	+	+	+	++
Str. viridans I (NCTC 3166)	±	±	±	±
C. diphtheriae (Park-William 8)	++	++	++	++

[a]From Kamat, V. N. *et al.* (109), Ditch plate method used.
[b]+, indicates action; ±, indicates partial action; −, indicates no action.

TABLE 9. *Antibacterial activity of C. roseus extracts by agar cup-plate method.*[a]

Organism	Aq. ext. (1: 4)	Fraction C	Fraction B	Fraction A	Total alkaloids
S. aureus	14.0[b]	24.4	20.4	17.7	21.0
B. coli	19.5	——	16.2	14.0	16.0
V. cholera	19.0	18.1	14.1	16.3	——
S. paratyphi A	nil	nil	nil	nil	nil
S. paratyphi B	14.0	nil	nil	nil	17.2
S. typhi	nil	14.0	nil	nil	nil
S. flexneri	nil	nil	nil	nil	nil
S. shigella	16.8	19.1	14.6	19.3	14.3

[a]From Neogi, N. C. and M. C. Bhatia (125).
[b]Indicates diameter of zone of inhibition in mm. Controls using sterile buffer gave no inhibition.

TABLE 10. *Antibacterial spectrum of C. roseus root bark alkaloids by serial tube dilution method.*[a]

Organism	Concentration of total alkaloids as hydrochloride in μg/ml				
	500	400	300	200	100
Esch. coli	++[b]	++	++	++	++
Sh. dysenteriae Flexner	++	++	++	++	++
Sal. typhosa	++	++	++	++	++
Strep. pyogenes	++	++	++	++	++
V. cholera	−	−	−	+	++
M. pyogenes var. *aureus*	−	−	−	−	++

[a]From Chopra, I. C. *et al.* (151).
[b]++, full growth as compared to control; +, partial growth; −, no growth.

and in 8 rabbits induced to hyperglycemia by the administration of 2 g/kg of glucose orally, little activity was observed.

Hano and Maj (159) have investigated the pharmacologic effects of an acidic aqueous extract of *V. minor*. In this study they injected the extract into the marginal ear vein of anesthetized, fasting rabbits and then determined blood glucose levels after 15, 60, 120 and 180 minutes. The results indicated that this extract decreased the blood glucose level to about 40 per cent of normal for at least 3 hours in all 5 rabbits used in the study.

Anti-malarial Activity of the Periwinkles.—*C. roseus* is the only member of this group reported to have been used as an anti-malarial drug (31,50). Spencer *et al.* (169) have reported on the screening of some 600 different plants, representing 126 plant families for anti-malarial activity. *C. roseus* was included in this study and aqueous extracts were found to exert moderate activity in oral doses equivalent to 4.42 g/kg utilizing trophozoite induced *Plasmodium gallinaceum* infections in white Leghorn chicks. Alkaloid extracts of this same plant produced slight activity at oral doses of 0.4 g/kg.

Anti-microbial Properties of the Periwinkles.—Several reports have been recorded in the literature concerning the use of the Periwinkles as folk remedies in

TABLE 11. *Anthelmintic activity of alkaloid fractions of C. roseus.*[a]

Fraction	Concentration	Time to kill ½ the no. of earthworms (min)	Time to kill all earthworms (min)	No. recovered life after 24 hours
Ether-soluble A	1–1000	40	55	nil
	1–5000	130	172	nil
	1–10,000	223	282	nil
Chloroform-soluble B	1–1000	59	72	nil
	1–5000	157	201	nil
	1–10,000	241	317	nil
Water-soluble C	1–500	Only 2 died after 6 hours.		
	1–1000	None died in 6 hours.		
Water		None died in 6 hours.		
Propylene glycol (5 ml) and water (4 ml)		None died in 6 hours.		
Santonin	1–1000	221	286	nil

[a]From Neogi, N. C. and M. C. Bhatia (125).

the treatment of microbial diseases. These diseases include dysentery (18,50), skin infections (50) and the diseases for which "cure-all" properties of the Periwinkles have been recorded in France (31,39). In addition, Dewey (148) suggests the use of *V. minor* tincture in homeopathic medicine as an aid in certain skin and scalp eruptions but he did not indicate whether these were actually infectious conditions.

Hays (149) and Sanders *et al.* (150) have screened the fresh juice of aqueous extracts of *V. minor* plants against a number of pathogenic organisms and found no significant inhibitory action by the cup-plate method. Kamat *et al.* (109) have tested vindoline tartrate and several different solvent extracts of *C. roseus* leaves against 13 strains of bacteria. Ethanol- and chloroform-soluble material from the leaves was found to contain significant anti-microbial activity against 8 of the 13 strains. Since all of the active solutions produced strong positive alkaloid tests, different alkaloid extracts were prepared and the activity of three of these extracts, together with vindoline tartrate, was again tested against the 8 susceptible strains of bacteria. The water-soluble alkaloid extract (fraction III) produced the highest degree of activity (table 8). Neogi and Bhatia (125) have

also tested several different solvent fractions of *C. roseus* whole plants against a number of enteric pathogens normally associated with diarrhea. Several of these extracts produced activity against 4 of 8 strains of test organisms used (table 9). Chopra *et al.* (151) utilized the serial-tube dilution method and tested the total alkaloid hydrochlorides of *C. roseus* root bark against several pathogens and noted inhibition of *M. pyogenes* var. *aureus* and *V. cholera* (table 10).

Anthelmintic Properties of Catharanthus Roseus.—*C. roseus* (61), *C. lanceus* (62), and *V. minor* (31) extracts have been reported to be useful as vermifuges. This prompted Neogi and Bhatia (125) to test three alkaloid fractions obtained from *C. roseus* for anthelmintic activity. The ether- and chloroform-soluble alkaloid fractions were more effective in killing earthworms than was santonin in equivalent doses (table 11).

Anti-cancer Properties of the Periwinkles.—The leukopenic action of extracts from *C. roseus* was first noted in a preliminary report by Beer (170) in 1955, who isolated an active alkaloid fraction from this plant which, by paper chromatographic studies, was found to contain at least 5 alkaloids. Only one of these 5 alkaloids exhibited leukopenic activity in rats. In 1957, Cutts (171) also reported on the effects of extracts of *C. roseus* on hematopoeisis in rats and noted results similar to those produced by aminopterin under the same conditions. The first report concerning the actual isolation of a leukopenic crystalline alkaloid was published in 1958 by Noble, Beer and Cutts (1). In studying extracts of this plant for hypoglycemic activity they observed a delayed toxicity in laboratory animals which prompted them to further investigate the cause of death. After autopsy, the rats used in this study were found to have died as a result of a *Pseudomonas* infection. After culturing the *C. roseus* extract and finding it free from organisms, the immune mechanism of injected rats was studied in an effort to ascertain the possibility of death due to opportunist infection by pseudomonads found as normal inhabitants of animal quarters. Extracts of this plant were then demonstrated in rats to produce an initial leukocytosis after injection, followed by a severe leukopenia lasting several days prior to returning to normal.

Since this depressant activity on leukocytes suggested a possible use of the plant or its alkaloids in the treatment of leukemia, further studies were conducted with the successful isolation of one of the leukopenic alkaloids which was named vincaleukoblastine (*VLB*). Crude material isolated during this investigation was also tested against transplantable mammary adenocarcinoma in DBA/JAX mice and against a transplantable sarcoma in the rat and was found to exhibit definite carcinostatic properties.

VLB has been demonstrated by Noble *et al.* to have antitumor activity against L1210 and P1534 leukemia transplanted in BDV hybrid mice, AKR leukemia transplanted in AK mice, and sarcoma 180 in Swiss mice (172). Further biological properties of this alkaloid have been reported by Cutts *et al.* (173) including the depressant activity against the Ehrlich ascites tumor in Swiss mice and the C3H mammary tumor. Also studied was the effect of hourly, daily and delayed injections of *VLB* on implanted tumors. Certain AK mice which showed indefinite survival following treatment with *VLB* were re-inoculated with 6 to 8 times the number of AK tumor inoculi necessary to produce leukemic deaths in control animals. These mice survived in excess of 250 days. When administered orally to rats, *VLB* had little or no leukopenic activity and when certain crude fractions were given by mouth, severe convulsions and death sometimes occurred. These investigators are of the opinion that *VLB* is only one of the active substances present in *C. roseus*.

Johnson *et al.* (174) have studied the activity of crude alkaloid fractions from *C. roseus* as well as that of *VLB* and leurosine, against P1534 leukemia in DBA 2 mice. In addition, *VLB* in combination with other antitumor drugs was studied in similar animals. These investigators reported that although leurosine does

show a retardation of the P1534 leukemia, it is a compound with a lower order of activity and is less consistent than *VLB*. Catharanthine and vindoline were shown to be devoid of P1534 leukemia activity in these tests.

Human choriocarcinoma, maintained in the hamster cheek-pouch, was inhibited by crude extracts of *C. roseus*, *VLB* and leurosine, while virosine, lochnerine, perivine, catharanthine and vindoline were ineffective (175). In these studies *VLB* and leurosine were not observed to exert significant toxicity.

Hertz, *et al.* have reported on the effective clinical use of *VLB* in treating methotrexate-resistant metastatic choriocarcinoma and other related trophoblastic tumors in women (3). Other reports have appeared in the literature concerning the clinical evaluation of *VLB* in treating Hodgkin's disease, acute leukemia, leukosarcoma, chronic myeloid leukemia, lymphosarcoma, giant follicle lymphoma, malignant thymoma, retinoblastoma with metastases, melanocarcinoma, acute monocytic leukemia, acute lymphocytic leukemia etc. (2,176–180). Although this new oncolytic agent has shown striking results in patients with many forms of malignant disease, *VLB* is primarily an agent for the treatment of Hodgkin's disease and choriocarcinoma. Undoubtedly further clinical evaluation will substantiate its use in other forms of cancer in the future.

To date, the exact mechanism of action of *VLB* has not been determined but *in vivo* studies concerning the effects of this drug on dividing cells (181), as well as *in vitro* studies of *VLB* effects on mitosis (182) indicate that it appears to be a spindle inhibitor and as such produces C-mitotic cell changes in addition to preventing cell division at metaphase.

Vincaleukoblastine is currently available for general use by physicians[5] in the treatment of Hodgkin's disease and choriocarcinoma but thus far is only thought of as a palliative and not as a cure. From an examination of published data concerning the activity of *VLB* and crude extracts of *C. roseus* it appears that perhaps another agent might be isolated with similar or perhaps more remarkable biological activity than *VLB*.

Other species of *Catharanthus* have not been reported in the literature with respect to possible anti-cancer evaluation, however, McKenna has published the results of screening 498 plants for cancer inhibition and *V. minor* was included. It did not exert anti-cancer properties in their tests (183).

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[5]Available as Velban®-Eli Lilly and Co. (Vinblastine sulfate).

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Peppermint and Spearmint Tissue Cultures. I. Callus Formation and Submerged Culture

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A comparatively recent application of plant tissue culture is the production or biosynthesis of useful plant constituents by normal appearing plant tissue cultures (1), by callus tissues grown on a solid medium (2), or by plant tissues and single cells growing in a liquid submerged culture (3). Alkaloids have been biosynthesized by excised root cultures (1,2,4,5), by callus tissues (2) and by Datura crown gall tissues (6). Although large quantities of various plant tissues have been obtained by liquid submerged culture, none have yet been reported to economically produce a useful plant constituent (7).

It was reported by Audus (8) that treatment of peppermint seedlings, with dilute solutions of naphthyleneacetic acid (*NAA*) often brought about a 30–50 per cent increase in the peppermint oil content of mature plants. Maciejewska-Potapczykowa and Kaminska (9) soaked peppermint seedlings in a solution of aminonaphthaleneacetic acid and after soil planting observed an increase in oil content of the leaves. Steward *et al.* (10) investigated the effects of day length and mineral nutrition on the nitrogen metabolism of peppermint by growing cuttings in water culture in a greenhouse.

Tissue cultures of volatile oil-producing plants have been successfully obtained from the integment tissue surrounding citrus ovules (11) and from the excised juice vesicles of the lemon (12). Neither of these reports made reference to the presence or absence of volatile oil in the cultures. No reference is made to the tissue culture of Labiates by Gautheret (13).

Callus tissue is generally acknowledged to be necessary in order to produce a submerged culture of a plant tissue (7,14,15). Callus tissue of normal sunflower stem (16) and carrot tissue (17) have been induced by indole acetic acid (*IAA*); of carrot root tissue (18) and kidney bean stem tissue (19) by 2,4-dichlorophenoxyacetic acid (*2,4–D*) and of artichoke tuber tissue (18) by 2–benzothiazoleacetic acid (*BTOA*).

Nutritional supplements, such as cocoanut milk (15,18), casein hydrolysate (18,20), malt extract (7) etc., or a medium containing specified amino acids (21) have been shown to facilitate callus induction and growth in certain tissues.

In our preliminary experiments, it was observed that vitamin-free, enzyme-hydrolyzed, casein hydrolysate inhibited the growth of peppermint and spearmint stems and seedlings. It was also observed that *BTOA* in concentrations greater than 1 ppm and *2,4–D* in various concentrations, were more effective callus-inducing agents than *IAA* or *NAA*. The effects of various concentrations of *2,4-D* and *BTOA* on peppermint and spearmint stems and seedlings are reported.

Prepared sections of peppermint and spearmint normal stems and 2,4–D or *BTOA*-treated stems were studied to determine the origin and the nature of the callus tissue.

The growth yield and the *p*H changes occurring in a four-week period of peppermint submerged cultures are reported.

This publication represents the first of a series of studies that will attempt to determine whether or not tissue cultures of peppermint and spearmint plants can biosynthesize useful aromatic constituents.

[1]This work is based on portions of a thesis to be submitted by M. L. Lin to the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the M. S. degree.

EXPERIMENTAL

Sources of Plant Materials.—Plants[2] of *Mentha piperita* L. and *Mentha spicata* L., used for stem tissue cultures, were maintained in the Drug Plant Greenhouse, University of Nebraska. About 50 per cent of the peppermint seeds, and about 30 per cent of the spearmint seeds,[3] used for seedlings, germinated in about five days.

Preparation of Media.—Media containing 0.7 per cent agar were used for both stems and seedlings in the following variations: (*i*) basal medium (table 1); (*ii*) basal medium with *BTOA*, 2, 6, 10, and 14 ppm; (*iii*) basal medium with *2,4–D*, 2, 6, 10, and 14 ppm; (*iv*) basal medium with *BTOA* and *2,4–D* in the following respective ppm concentrations: 2 and 2; 2 and 6; 6 and 2. Media were prepared with distilled water redistilled from glass and 15 per cent cocoanut milk when indicated as present in the medium. Media were adjusted to *p*H 5.8 with 10 per cent potassium hydroxide before autoclaving. Approximately 10 ml of each medium was added to a one-ounce wide-mouth prescription bottle, covered with a plastic cap, and autoclaved at 130° C for 15 min. All auxin stock solutions were used on the same day of their preparation.

TABLE 1. *Basal Medium*[a]

	mg/liter
Modified Murashige's inorganic salts[b]	
KNO_3	950.0
NH_4NO_3	720.0
$MgSO_4 \cdot 7H_2O$	185.0
$CaCl_2$	166.0
KH_2PO_4	68.0
$MnSO_4 \cdot H_2O$	7.0
$ZnSO_4 \cdot 7H_2O$	4.05
H_3BO_3	2.4
KI	0.375
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0925
$CuSO_4 \cdot 5H_2O$	0.01
Iron ethylenediamine tetraacetic Acid[c]	39.75
Vitamin solution[d]	
Biotin	1.0
Choline	1.0
Folic acid	1.0
Nicotinamide	1.0
Pantothenic acid	1.0
Pyridoxal	1.0
Thiamine	1.0
Riboflavin	0.1
Inositol	5,000.0
Sucrose	20,000.0

[a]Media were prepared with inorganic chemicals of Reagent Grade and organic chemicals from either Nutritional Biochemical Corp., Cleveland, Ohio or Calbiochem, Los Angeles, Calif.

[b]Unpublished formula of Dr. Toshio Murashige, Hawaii Agricultural Experiment Station, Honolulu.

[c]Perma Green Iron 135, Refined Products Corp., Lyndhurst, N. J.

[d]Eagle's Vitamin Solution, Hyland Laboratories, Los Angeles, Calif.

[2]Nurserymen's Exchange, San Francisco, Calif.

[3]George W. Park Seed Company, Greenwood, So. Car.

Selection and Sterilization of Stems and Seeds.—Defoliated stems, containing five nodes from the apex, were sterilized with 2.5 per cent sodium hypochlorite solution[4] in vacuum for approximately 15 min. The stems were washed six times with sterile distilled water and transferred to a sterile Petri dish containing 10 ml sterile distilled water. The third and fourth nodes from the apex of the stem were cut into approximately 1 cm lengths with the nodes in the center of each section. The stems were transferred by placing the base of the stem partially into the medium; two sterile stem sections were placed in each of three prescription vials. Four replicates were made for each medium tested. The incidence of contamination was about 30 per cent.

Seeds were sterilized, washed, and transferred to a sterile Petri dish containing 10 ml sterile distilled water in a similar manner as for the stems. After germination and growth to a length of 1 to 1.5 cm in approximately three days, they were transferred to the appropriate medium. Two sterile seedlings were placed in each of three prescription vials. Two replicates were made for each medium tested. The incidence of contamination was about 5 per cent.

Preparation of Histological Sections.—Normal stem tissues and stems growing one month on basal medium with cocoanut milk and *BTOA* (6 ppm) or *2,4–D* (6 ppm) were killed with formalin-acetic acid-alcohol solution. The materials were dehydrated according to a *n*-butanol schedule (22), embedded in "Tissuemat"[5] (mp 60–63°C), sectioned at 10μ with a rotary microtome, and stained with safranin and fast green.

Submerged Culture.—Segments of two-month old peppermint callus tissue, approximately 1 cm diameter, were transferred to 150 ml of experimental media without agar in a 500 ml Erlenmeyer flask. The flasks were placed on a shaker,[6] rotating 130 rpm in a 2.5-cm circle, at room temperature. The fourth liquid subculture of the tissue was studied for growth and *p*H change over a four-week period. All transfers were made into each Erlenmeyer flask twice with a porous platinum wire transfer cup 1.2 cm in diameter and height. The average dry weight of the tissue transferred to each flask was 15 mg which was equivalent to 75 mg fresh weight.

Twenty flasks were initially prepared and inoculated and four were examined at intervals for the amount of growth and *p*H change in the medium.

RESULTS AND DISCUSSION

Control Cultivation of Stems and Seeds.—Control stems of both peppermint and spearmint plants on the basal medium often formed shoot buds and roots at the nodes. In time, normal-appearing plants developed which had glandular cells and hairs on both leaf and stem surfaces. Although the shoot buds and roots from the stem grew well on the basal medium, they never grew as vigorously or as large as plants grown in soil. The growth of the original stem tissue on the medium was always retarded.

Control seedlings of both plants developed normal-appearing roots, stems, and leaves on the basal medium.

Effects of Cocoanut Milk.—The addition of cocoanut milk to the basal medium did not stimulate callus formation from either stems or seedlings. However, if cocoanut milk was added to an auxin-containing medium, the callus tissue formed was harder, lighter in color, and did not change to a brown color as rapidly as callus formed on the auxin-medium without cocoanut milk.

Cocoanut milk significantly improved callus growth of peppermint and spearmint seedlings initiated by *2,4-D*, and slightly improved callus growth initiated by *BTOA*.

[4]Purex Corporation, Ltd., South Gate, Calif.
[5]Fisher Scientific Company, Pittsburgh, Pa.
[6]Model V, rotory action shaker, New Brunswick Scientific Company, N. J.

Effects of BTOA.—The BTOA-induced callus from either peppermint or spearmint stems were similar and always contained short roots with many root hairs on their surface. Adventitious roots that touched the medium often formed callus tissue.

Approximately 80 per cent of the stems grown on the lower *BTOA* concentration (2 ppm) formed callus tissues but these tissues did not subculture as well as stem callus tissues grown at a higher concentration (6 ppm). No particular advantage was observed in the use of still higher concentrations of *BTOA* (10 and 14 ppm) for inducing stem callus tissues.

Initiation and growth of callus from seedlings was greater with the increased concentrations of *BTOA*. The effect of *BTOA* on shoots and roots from the seeds was similar to that observed on the stems and as described in previous paragraphs. The concentration of *BTOA* recommended for inducing seedlings to produce callus is 10 ppm.

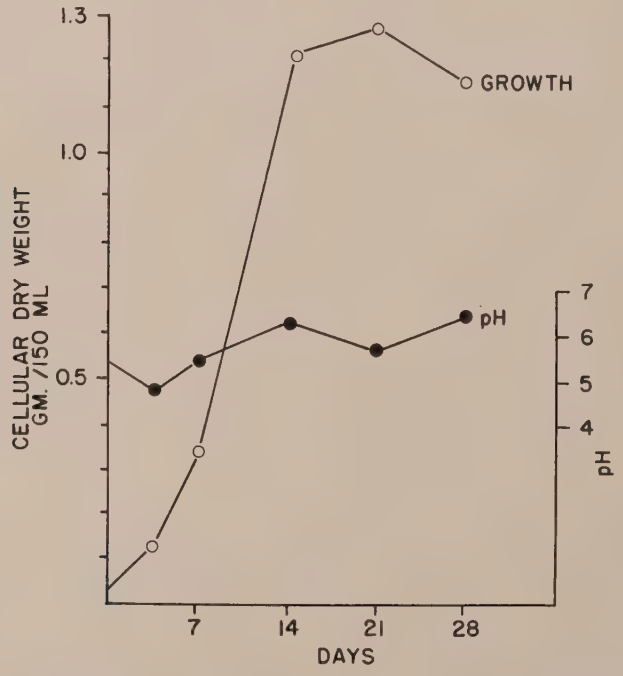

FIG. 1. *Cellular growth and medium pH change in peppermint submerged cultures.*

Effects of 2,4–D.—The stem callus tissue induced by *2,4–D* (2,6,10 and 14 ppm) was similar to that formed by *BTOA*, but neither the stem nor the callus contained shoots or roots. This observation is contrary to the effects of root initiation by *2,4–D* on tomato stems (19) and rice stems (23). The concentration of *2,4–D* recommended for inducing stem callus tissues is 6 ppm.

The auxin *2,4–D* has been reported to inhibit the growth of soybean seedlings (24). The growth of seedlings of both peppermint and spearmint plants was inhibited by *2,4–D* and only very small callus tissues grew.

Auxin Interaction.—The combination of *BTOA* (2 ppm) with *2,4–D* (2 ppm) produced a stem callus similar to that induced by *2,4–D* (6 ppm) alone. No particular advantage was observed in the use of an auxin combination for stem callus growth. However, this same auxin combination induced a seed callus

which did not produce roots or shoots and grew better than that induced by *2,4–D* alone.

Histological Effects.—In the majority of sections observed, the auxin *BTOA* (6 ppm) seemed to cause a proliferation of cells in the tissues located in close proximity to the vascular tissue, often originating from either the cambium, endodermis, or cortex. Adventitious roots were observed to originate from the pericycle at the interfasicular area, and they appeared quite similar to those roots formed by *2,4–D* in the rice plant (23). Xylem cells were frequently seen irregularly distributed throughout the callus tissues.

Similar types of xylem cells have been reported induced by *IAA* in tobacco stem callus tissues (25) and callus tissue from citrus ovules (11), and also by *2,4–D* in carrot phloem callus (26).

The histology of the stem was affected by *2,4–D* in the same way as *BTOA*, except it did not cause pronounced root formation. Although xylem cells were not observed, concentric meristematic tissues were distributed randomly throughout the callus tissue.

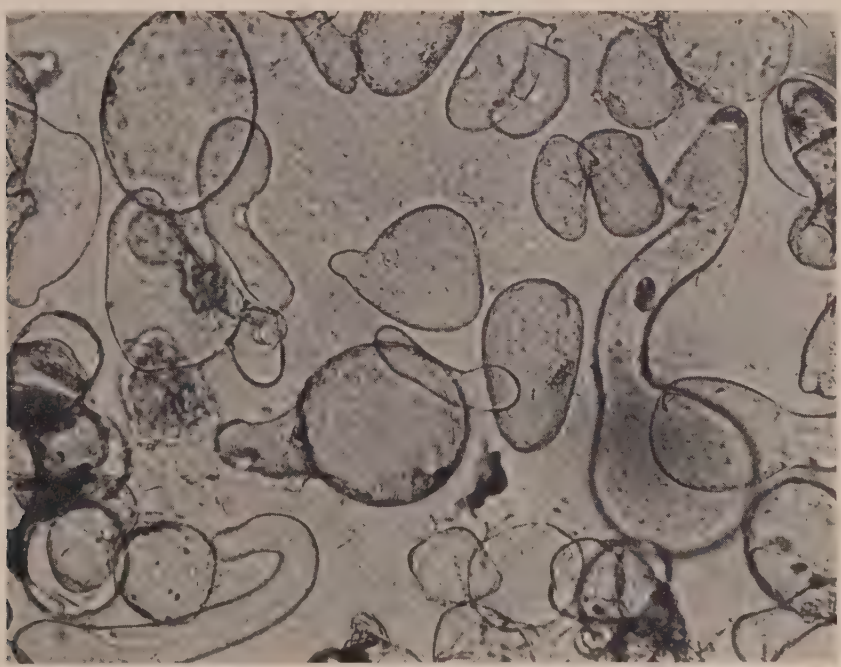

FIG. 2. *Cellular growth originated from peppermint stem callus, observed at the end of thirty-one days of submerged culture.*

Submerged Culture.—Cellular growth was obtained in the liquid basal medium without cocoanut milk, but containing *2,4–D* (2 ppm). Free cellular growth was very poor in the liquid basal medium containing *BTOA* (2 ppm) or 15 per cent cocoanut milk. Growth and *p*H changes during a four-week growth period are shown in figure 1.

Large aggregates of cells similar to those described by Nickell and Tulecke (7) were often found in the first two weeks, with an observed increase of occurrence of single cells and small aggregates of cells (2–7 cells) during the latter two weeks of the growth cycle. Figure 2 shows giant cells and variation of cell shapes frequently observed in the liquid cultures. When peppermint cells from the

submerged culture were returned to the surface of the original growth medium containing agar, callus cells formed as a mat.

SUMMARY AND CONCLUSIONS

The use of 2,4–dichlorophenoxyacetic acid and 2–benzothiazoleacetic acid in a modified Murashige's medium induced callus tissue in either peppermint or spearmint stems or seedlings. An auxin concentration of 6 ppm of *2,4–D* is recommended for the formation and subculture of stem callus tissues, and 2 ppm of *2,4–D* with 2 ppm of *BTOA* for the seedling callus. Cocoanut milk was observed to have beneficial growth effects for callus tissues of both mint species.

Stem callus tissue induced by *BTOA* (6 ppm) frequently contained vascular elements, whereas that induced by *2,4–D* (6 ppm) often contained concentric meristematic tissues.

A modified Murashige's medium containing *2,4–D* (2 ppm) was found to enable peppermint callus tissue to grow as free cells in submerged culture.

ACKNOWLEDGMENTS

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Gibberellin Effects on Carbohydrate and Glycoside Content and Growth of Digitalis lanata

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Gibberellin effects on higher plants have been observed from many perspectives. Studies have been performed in an effort to determine the effect of gibberellic acid (*GA*) on the concentration of various plant constituents with variable results reported. Alkaloid content was reported by Sciuchetti *et al.* (1–3) to be lower in treated *Datura stramonium* and *Atropa belladonna*. Reduced concentrations were noted especially in the shoots of treated plants. Similar results were noted by Burk and Tso (4) after treatment of *Nicotiana* spp. A decrease in rutin content of treated buckwheat was reported by Sayed and Beal (5). However, the same authors indicated that the glycoside concentration in *Digitalis purpurea* was increased following treatment with *GA* (6).

The effect on primary plant constituents has also been observed. Total sugar was reported to be reduced significantly in Kentucky bluegrass (7) and sugar beets (8) treated with *GA*. However, the *GA* treatment of *Digitalis purpurea* (6) resulted in an increase in total sugar content.

The biosynthesis of digitalis glycosides has been suggested to be closely interrelated with carbohydrate metabolism. Tsao and Youngken (9) have reported the effects on digitalis glycoside production by other compounds influencing carbohydrate metabolism. The results of their work fit the expected pattern assuming such an interrelationship.

EXPERIMENTAL

Growth Studies.—Volunteer *Digitalis lanata* Ehrh. seedlings from the School of Pharmacy drug garden were transplanted into flats on May 2, 1959. They were transplanted a month later into one-gallon containers filled with a sandy loam mixture. One hundred sixty-eight of the potted plants were placed in rows in a shaded garden area. Each container was fertilized with approximately 8 g of a complete fertilizer[2] at this time and again halfway through the growth study. Slug and insect damage was minimized by the use of slug pellets[3] and by the application of 4 per cent malathion dust respectively at weekly intervals. The plants were divided randomly into three groups: (*i*) control; (*ii*) high–*GA* dosage; and (*iii*) low–*GA* dosage.

Weekly treatments and height measurements began on July 7, 1959 and continued for sixteen weeks. The high-dosage group received weekly doses of 50 μg *GA*[4], per plant, in 0.01 ml water delivered from a micro-pipette onto the youngest leaf. Each plant in the low-dosage group received weekly doses of 10 μg *GA*. In the event of rain on a treatment day, all plants were covered with a tent-like structure of polyethylene sheeting. Measurements were made from the

[1]This manuscript was abstracted from a thesis submitted to the Graduate School of Oregon State University by Stephen D. Burton in partial fulfillment of the requirements for the degree of Master of Arts. Present address: School of Pharmacy, The University of Connecticut, Storrs.

[2]Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis: 5% of total nitrogen, 3% available phosphate, 2% available potash).

[3]Snarol, Boyle-Midway, Inc., Los Angeles, Calif. Active ingredients: tricalcium arsenate —5% and metaldehyde—3.15%.

[4]The *GA* (88.9% *GA*, Lilly, #0757–P–60626) was supplied by Dr. Edwin F. Alder, Agricultural Research Center, Eli Lilly and Company, Greenfield, Ind.

point of emergence of the plant from the soil to the highest point reached while gently bunching the leaves. The average observed heights at weekly intervals are shown in figure 1.

Eight plants from each of the treated and control groups were harvested every twenty days. Fresh and dry weight determinations were made on the roots and shoots[5] (table 1) of individual plants. The plant parts were dried in a circulating hot-air dryer at approximately 48° for 24 hrs, and powdered (no. 60 mesh) in a Wiley mill. For chemical assay the powders were pooled at each harvest according to group and plant part, except at zero time, in which case, due to the small size of the plants, all groups were pooled.

Glycoside Determination.—A modification of Soos' method (10) was used to extract 250-mg samples of dried, powdered leaves. Each sample was shaken with 25 ml of 20 per cent ethanol for 12 hrs on a wrist-action shaker at moderate speed. The extract was filtered and the residue washed five times with distilled water to bring the filtrate volume to approximately 45 ml. This solution was extracted

TABLE 1. *Average weight of Digitalis plant parts*

Treatment	Time in weeks	Shoots			Roots			Total plant		
		Fresh g	Dry g	% of control dry weight	Fresh g	Dry g	% of control dry weight	Fresh g	Dry g	% of control dry weight
Control	0	0.296	0.043	——	0.026	0.011	——	0.322	0.054	——
	4	1.717	0.180	——	0.388	0.029	——	2.105	0.209	——
	8	5.800	0.677	——	0.860	0.100	——	6.660	0.777	——
	12	10.760	1.490	——	1.610	0.200	——	12.370	1.690	——
	16	16.370	2.750	——	3.110	0.420	——	19.480	3.170	——
50 μg *GA*	0	0.204	0.035	81.4	0.014	0.008	72.7	0.218	0.043	79.6
	4	2.929	0.298	165.6	0.694	0.035	120.7	3.623	0.333	159.3
	8	12.880	1.535	226.8	1.510	0.200	200.0	14.390	1.735	223.3
	12	13.310	1.720	115.4	1.170	0.170	85.0	14.480	1.890	111.8
	16	13.080[a]	2.170[a]	78.9	1.370	0.230	54.8	14.450	2.400	75.7
10 μg *GA*	0	0.263	0.046	107.0	0.018	0.009	81.8	0.281	0.054	100.0
	4	2.184	0.230	127.8	0.355	0.025	86.2	2.539	0.255	122.0
	8	9.450	1.207	178.3	0.950	0.104	104.0	10.400	1.311	168.7
	12	16.200	2.310	155.0	2.040	0.290	114.5	18.240	2.600	153.8
	16	22.640[a]	3.490[a]	127.0	2.510	0.390	92.9	25.150	3.880	122.4

[a]Bolting had occurred at this harvest. For the purpose of the growth study the weights of the stems were included in the weights of the shoots. The stem fresh weight (ave) of the 50-μg group was 0.75 g (dry weight, 0.15 g); for the 10-μg group the respective figures were 1.37 g (0.25 g).

three times with 15-ml portions of chloroform. The combined chloroform solution was evaporated in a 50-ml Erlenmeyer flask (glass-stoppered) to a low volume on a hot water bath and before analysis completely dried on a bath maintained at 50 to 55° C.

The samples were assayed by a modification of the National Formulary X procedure for Lanatoside C tablets (11). The procedure was modified by carrying out the reaction in the glass-stoppered flask in which the sample had been evaporated and by adding glass beads to facilitate solution of the sample. The concentration of glycosides[6] was determined colorimetrically.[7] The results of these analyses are shown in table 2.

[5]At the final harvest a definite stem was observed in the treated plants and these plants were divided into roots, stems, and tops.

[6]The color developed in this reaction is associated with digitoxisidic glycosides.

[7]Bausch and Lomb Spectronic 20 colorimeter.

Carbohydrate Determination.—The procedure for extraction of total sugar was adapted from that of McCready (12). A 250-mg sample of leaf material was shaken with 15 ml of hot, neutral (to litmus), 80 per cent ethanol for 30 min on

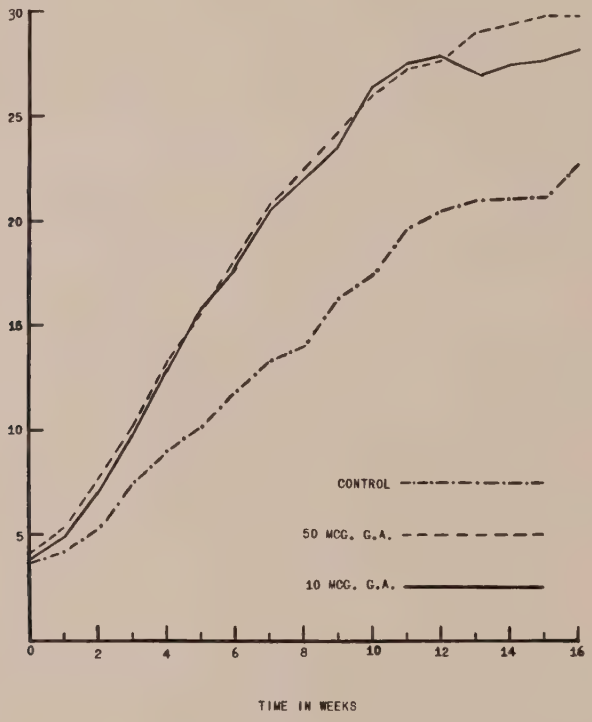

FIG. 1. *Average height (cm) of digitalis plants.*

TABLE 2. *Analysis of glycosides in Digitalis shoots.*

Treatment	Time in weeks	Total glycosides[a]		Total glycosides per shoot[a]	
		mg/g	% of control	mg	% of control
Control	0	3.48	——	0.14	——
	4	4.28	——	0.77	——
	8	4.72	——	3.20	——
	12	5.12	——	7.63	——
	16	6.08	——	16.72	——
50 μg GA	4	4.40	102.8	1.31	170.1
	8	6.32	133.9	9.70	303.1
	12	4.36	85.2	7.50	98.3
	16	4.60	75.7	9.98	59.7
10 μg GA	4	4.12	96.3	0.95	123.4
	8	4.92	104.2	5.95	185.9
	12	5.72	111.7	13.21	173.1
	16	6.16	101.3	21.50	128.6

[a]Calculated from average dry weight.

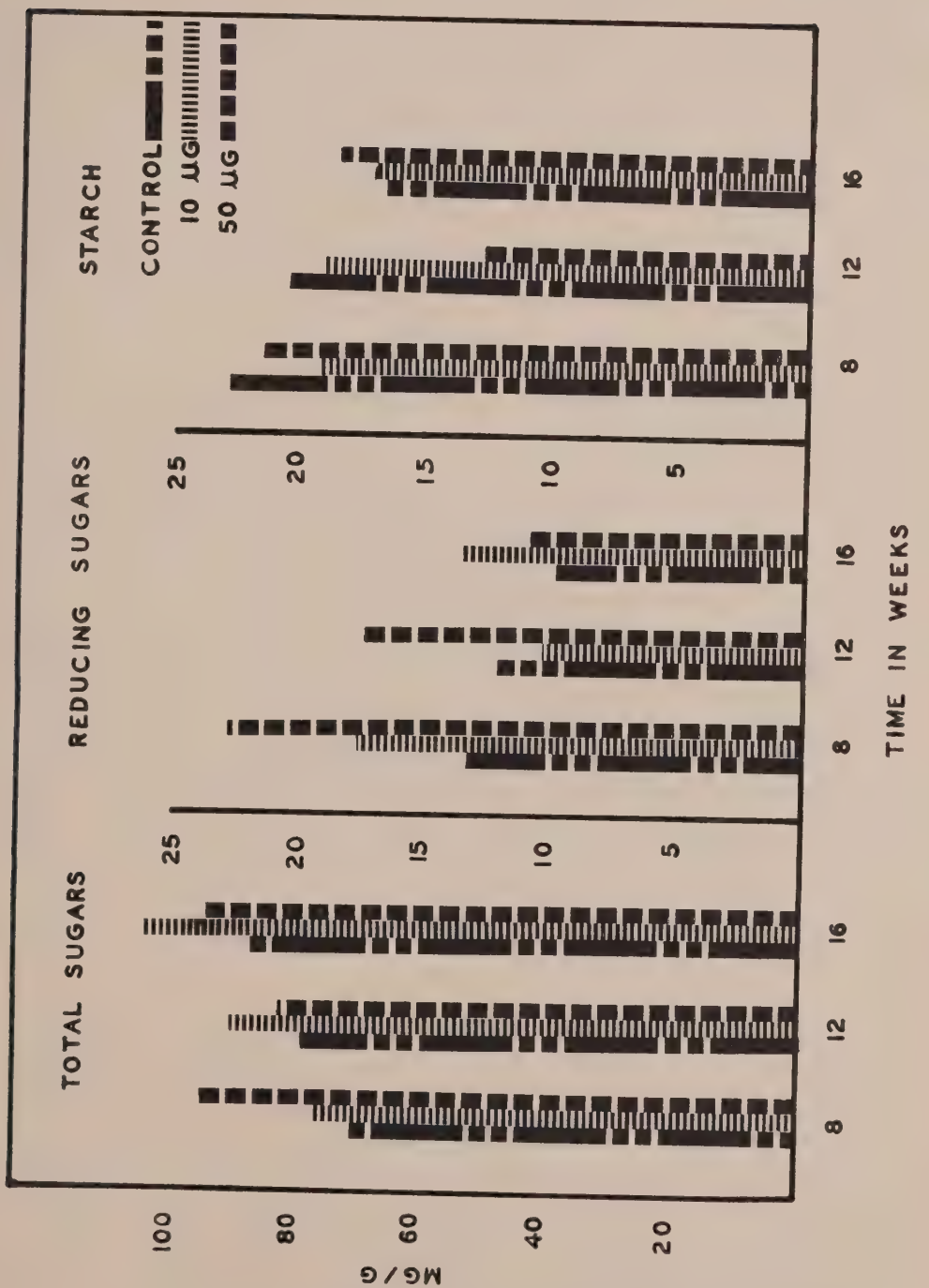

FIG. 2. *Carbohydrate concentration in Digitalis lanata shoots.*

the wrist-action shaker. The mixture was centrifuged and the supernatant liquid decanted into a 100–ml volumetric flask containing 25 ml of 15 per cent neutral lead acetate solution. The residue was washed three times with the hot ethanol solution and centrifuged. Each time the supernatant liquid was decanted into the flask containing lead acetate. Powdered sodium oxalate was added to remove the lead ions and the mixture was brought to the 100-ml mark with distilled water. The mixture was centrifuged and aliquots taken from the supernatant liquid.

Two-ml aliquots of the above were used for the determination of the reducing sugar content (fig. 2) according to the method of Nelson-Somogyi (13–14). Total sugar was determined by means of the anthrone-sulfuric acid color reaction (15) using 0.3–ml aliquots of the clarified extract. The concentrations are shown in figure 2.

The residue from the sugar extraction procedure was extracted and analyzed (16) for starch (amylose). The results of these analyses are presented in figure 2.

FIG. 3. *Effect of gibberellic acid on Digitalis lanata; plants at time of final harvest. A=50 μg GA; B=10 μg GA; C=control.*

DISCUSSION

Habit.—Typical gibberellin effects were noted in both groups of treated plants, such as development of narrower, more elongated, and slightly chlorotic leaves. The treated plants began bolting at the twelfth week and had developed definite stems by the final harvest (sixteenth week) while the control plants remained acaulescent (fig. 3).

Growth.—The increases in height and shoot weight in the 10–μg group followed a parallel trend throughout the growth study. Moreover, the average heights of the plants receiving 10–μg of *GA* were similar to those of the 50–μg group, the differences being considered insignificant. However, in the 50–μg group the weight of the shoots increased at a slower rate than did the height after exhibiting the greatest weight increase at the eighth week. At the final harvest, the weight of the shoots of the 50–μg group was only 75.7 per cent that of the control shoots, whereas the height was 130.7 per cent of the controls. This fact might be indicative

of an accumulative toxic effect resulting in a decreased ability to synthesize structural material at a rate sufficient to keep up with the stimulated vertical growth.

No significant effect on root growth was demonstrated by the 10-μg dose. However, the data obtained suggest an evanescent stimulation by the larger dose of *GA*, with inhibition of root growth, as the overall effect at this dosage level.

Glycoside Content.—The effect on glycoside production appeared to correlate more closely with the effect on growth than with the effect on carbohydrate content. The decrease in total glycoside per plant in the 50-μg group at the twelfth week indicates that the lower concentration of glycosides demonstrated by this group in the later stages of the study was not due to dilution. This reduction in content would not necessarily imply a breakdown of the steroidal aglycone but might be the result of hydrolysis of some of the glycoside fraction since the assay reaction is based on the digitoxose moiety. The total carbohydrate concentration at this stage was also greatly reduced from the level demonstrated at the eighth week. For the same period, the increase in height was greater and the increase in dry weight was less than in the controls. The apparent toxic action of *GA* at this dosage level may have resulted in a shortage of precursors in the synthesis of structural material.

The effects on weight increase and carbohydrate and glycoside content by the 10-μg dose did not follow a trend similar to that of the 50-μg dosage group. The overall effect of the 10-μg dose of *GA* appeared to be beneficial, causing an increase in growth rate and a more rapid maturation of the plants without drastically upsetting the metabolism.

SUMMARY

A study was made of the effects on the growth and concentration of glycoside and carbohydrate in *Digitalis lanata* Ehrh. induced by weekly doses of 10 and 50 μg of *GA*. Characterisitic gibberellin effects were observed in plants of both groups.

The heights of the treated plants were significantly increased with the peak effect noted at the eighth week. Bolting was induced between the twelfth and sixteenth weeks.

Both treatments demonstrated an increase in the dry weight of the shoots compared with the controls. The maximum effect was obtained at the eighth week. Only in the 50-μg group was the dry weight of the roots significantly altered, in which case, a significant reduction in root growth was noted.

The total glycoside content per shoot of the treated plants disclosed significant increases during the first eight weeks. For example, the 50-μg group had a threefold increase. At the final harvest, however, this group had about half the total glycoside content compared to the controls, whereas the 10-μg group had about a 30 per cent increase.

In general, the treatments resulted in an increased level of total sugar and of reducing sugar. Both groups exhibited a lower starch concentration than the controls at the eighth and twelfth week, but a slight increase was noted at the terminal harvest.

It was noted that the 50-μg dose appeared to be toxic to the plant after the eighth week whereas the lower dose gave primarily a beneficial effect.

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Studies on Spirostreptoid Millipeds.

VI. A Redescription of Trachystreptus cambaloides and Some Remarks on Its Classification

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Trachystreptus is one of O. F. Cook's numerous "footnote genera." In his outline of diplopod classification published in 1895 (1), Cook proposed the new family Trachystreptidae to receive two new genera from Africa: *Trachystreptus* and *Lophostreptus*. The former name was proposed as new for *T. cambaloides*, which was very briefly diagnosed in a footnote which served to validate, if not characterize, both the genus and species. Nobody, without specimens in hand, could possibly get more than a vague idea about the characters of this type species of a genus and family. *Lophostreptus* was based upon an equally obscure species which was originally named *Glyphiulus magnus* by Karsch.

In the following year, Cook elaborated his singular family in a paper entitled "The genera of Trachystreptidae" (2). Here five new genera were added to the original pair, and all seven brought together in the form of a key. Some commentary was also provided; a casual discussion of the family characters and remarks on certain East African species.

It can be appreciated that European workers with a preference for greater explicity in descriptions did not react favorably to the general subject of the Trachystreptidae! In 1909 (3), the Count von Attems reduced the group to a subfamily of the Spirostreptidae, as he could find nothing really distinctive in the gonopod structure of the genera which he studied, and in 1914 (4) he degraded it still further to the level of a tribe in the Spirostreptinae. Here the group has persisted somewhat precariously—even tribal status has been questioned—down to the present.

The status of the name Trachystreptidae rests, however, solely on the characters of *Trachystreptus cambaloides*, a species which nobody has ever been able to study carefully since the time of its proposal. In the Hamburg Museum Attems found two females, labeled as *cambaloides* by Cook, and incorrectly assumed them to be the types of the species. But the real type specimens are in the collections of the U. S. National Museum and I am glad to be able to publish a description and some drawings of important taxonomic characters. It may be years before enough material accumulates to warrant preparation of a revision of the African crested spirostreptoids, but knowledge of the type species of the family name will be of great usefulness at that time. The availability of such information at the present time may also be helpful to persons studying West African diplopods, and may prevent some needless synonymy.

Trachystreptus cambaloides Cook

Figures 1–6

Trachystreptus cambaloides Cook, 1895, Ann. New York Acad. Sci., **9:** 4; 1896, Brandtia, **13:** 55.—Attems, 1914, Zoologica, **25** (65/66): 146.

Lectotype.—Male, U. S. Nat. Mus. Myriapod Type No. D–576, from Mount Coffee, 17 miles northeast of Monrovia, Liberia, collected in 1894 by O. F. Cook. Chosen from two males in a vial labeled "Type" in Cook's handwriting.

Diagnosis.—Distinguishable from all other known trachystreptines by the combination of smooth sternites and paraprocts, excavation of the lower anterior part of the collum, and restriction of the segmental crests to the elevated caudal

portion of the metazonites. The species differs from all others in which males are known by the caudally located opening of the gonocoel and the broadly laminate exospermital portion of the telopodite.

Description of lectotype: Adult male with 36 segments, only the last one legless; maximum diameter 2.4 mm, length, about 26 mm.

Color altered by preservation, at present uniformly light bluish-gray overall; the legs, antennae, and lower half of face olive-green. With higher magnification the yellow spots of the metazonites are visible through the exoskeleton as testaceous circles between the longitudinal crests of most segments.

Head evenly convex, smooth and polished. Epicranial suture distinct, vertex with the same texture as frons, but finely and sparsely punctate. Labral margin nearly straight, the median notch distinct, with three low equal-sized teeth. Labral setae 8–8. Genal region distinctly impressed below and behind antennal sockets, forming a depression for the curiously bowed base of the second antennal article (fig. 1). Ventral and caudal edges of the antennal sockets margined; dorsal and cephalic edges immarginate but slightly overhanging base of the first article. Ocellaria rather large, subtriangular, their inner angles separated by a distance about equal to length of one ocellarium. Ocelli in five rows, decreasing in length ventrally, viz.: 10–10–7–4–2.

Antennae short (scarcely exceeding caudal edge of collum when laid back) and very robust, all articles except 2nd wider than long, and each very abruptly clavate (fig. 1). First article smooth, subhemispherical, its dorsal part concealed by the overhanging edge of the antennal socket, its ventral half narrowed and with a single, ventrally-directed seta. Second article slightly longer than its greatest width, projecting first ventrolaterad from the 1st, then abruptly bent caudodorsad at about a 35 degree angle and doubling in diameter, its dorsal and ventral surfaces set with numerous fine short setae. Articles 3–5 similar in size and shape, subtriangular, distinctly compressed, covered with fine sparse setae; article 5 considerably wider than long, 6 subglobose, 7 small, in the form of a short, flattened cylinder containing a small round distal opening which encloses the four small sensory cones. Articles 5 and 6 with small ovoid sensory pits on the outer distal surface, each containing about a dozen tiny sensory pegs. Interantennal isthmus equal to length of 2nd antennal article.

Gnathochilarium of the form typical of the order; each stipes with a small elevated calloused area just below the palps and with two apical setae on each side. Mentum with a transverse ridge near the base, behind which the surface is distinctly excavated.

Collum much broader than the head, evenly convex but heavily and densely pitted and punctate except near the anterior margin. The pitting becomes more accentuated near the caudal edge, the impressions elongating and grading into longitudinal grooves separated by the rather short but prominent crests in a transverse series along the margin. A large and conspicuous smooth ridge runs obliquely caudoventrad across each side of collum from level of the ocellaria; ventrad of this ridge the collum is deeply impressed and concave, forming a

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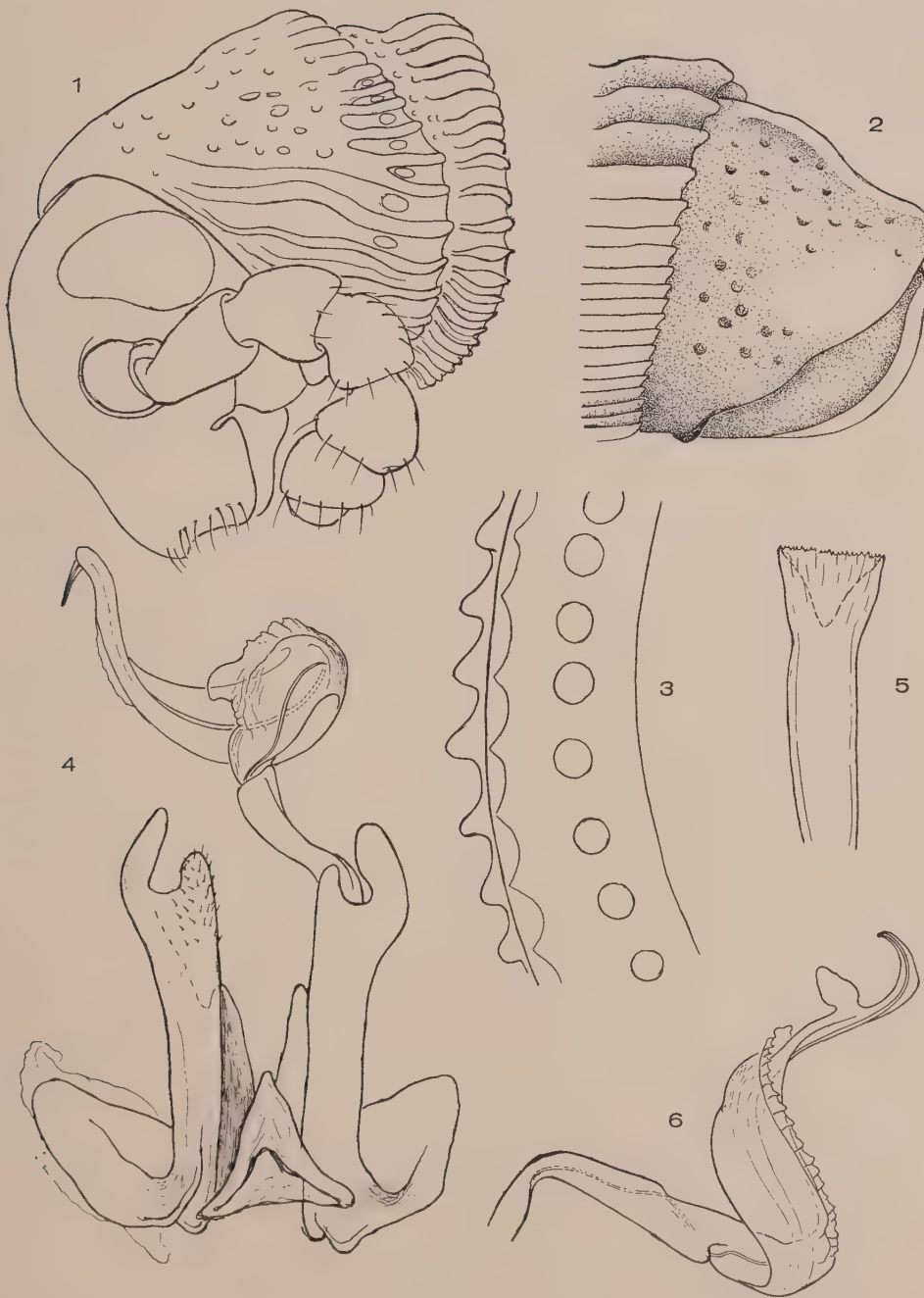
EXPLANATION OF FIGURES

FIG. 1–6. *Trachystreptus cambaloides Cook.* 1. *Head and first two body segments, anterior-lateral aspect.* 2. *Last two segments and paraprocts, lateral aspect.* 3. *Strip of metazonite of a midbody segment, viewed from the inside, to show size and distribution of the yellow spots.* 4. *Sternite and coxites of gonopods, anterior aspect, telopodite of the right gonopod also shown but not in its correct position (distorted by cover slip).* 5. *Prostatic stylet of coxite, enlarged.* 6. *Isolated telopodite of left gonopod, in a different aspect to show marginal fringe and subterminal lobe. Figs. 1–3 from lectotype, 4–6 from male topoparatype, Mount Coffee, Liberia.*


1
2
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6

depression for the accomodation of the antennae. Surface of this region generally finely striate, with a few small ridges on one side of the collum but not on the other.

First pair of legs as in most other spirostreptoids, composed of a coxosternum and five free podomeres plus a pretarsus, but the coxosternal sutures are distinct and visible, and the structure is medially suturate. Prefemora with elongate digitiform retrorse processes which insert between the gular and mental plates of the gnathochilarium.

Pleuron of 2nd segment strongly modified: the crested and ribbed metazonite strip continues without interruption to the apex of the inturned part of the segment, but the corresponding part of the prozonite is set off from the rest of that subsegment by a deep groove (visible only when the collum is drawn forward), which appears to accomodate the distinctly enlarged and thickened phragmal septum of the collum when the body is rolled.

Prozonites of body segments smooth, without any trace of transverse striae. Interzonal furrow broad and moderately deep, ornamented with numerous fine longitudinal striations, alternating numbers of which (more or less) continue onto the metazonite, there to increase in size and height. Each crest is notched or even obliterated about its midlength, the posterior half thus isolated becoming distinctly higher and larger than the anterior, particularly so on the dorsal part of the segments, and projecting slightly beyond caudal edge of metazonites. The dorsal crests (counted across the segment between the level of the ozopores) vary from 22 to 26, the larger numbers due to irregularly intercalated short crests. Sides of segments below ozopores with crests becoming increasingly smaller ventrad and difficult to count with accuracy, but usually about 16 between pore and sternite.

Ozopores large and conspicuous, beginning on segment 5, the peritreme consisting of the swollen anterior half of a lateral crest. All segmental ornamentation, including the peritremata, becomes accentuated on the caudal third of the body, but the lateral crests of segments 2 through 4 are likewise distinctly enlarged over those that follow.

Anal segment large and distinct, somewhat galeate, with a low but prominent median carina along the middle of its length. Dorsal surface of segment rough and pitted with irregular-sized punctures, these interspersed with smaller punctations which occur also on the median carina, but which grade off in size and abundance in going ventrad on the segment. Median projection of the caudal edge (epiproct) broad and fairly distinct, slightly overhanging the paraprocts in lateral aspect (fig. 2). Latter convex, smooth, their mesial edges set off by a deep, narrow, submarginal groove. The marginal areas thus set off meet to form an essentially flat surface, not an acute or re-entrant angle as is normal for most Diplopoda. Hypoproct very small and narrowly transverse, appearing to be partially fused to the broad ventral surface of the penultimate segment.

Legs of moderate size and length, the tarsi mostly visible beyond sides of body when viewed from above. Surface of podomeres polished and virtually glabrous (the setae restricted to a single apical ventral seta on each podomere except for the tarsus, which has two to four and a few microsetae on the dorsal side). Lengths of podomeres in decreasing order: 3–2–5–4–6–1, but all are virtually the same length, and none are especially clavate distally. Postfemora and tibiae with faint indications of apical ventral pads, or surficial openings for them, but no evidence of extrusibility can be observed.

Prosternites rectangular, elongate (about 50 per cent longer than wide), completely smooth, their stigmata very small and longitudinally ellipsoid, opening flush on the flat sternal surface. Metasternites small and short, about as long as wide, smooth, their stigmata larger, rounded, and opening near the leg sockets at the lateral edge of the sternite, with a corresponding emargination of eleva-

tion of the adjoining pleural edges. Pleurites of 7th segment produced mesad and in contact at the median line, but not in any degree fused.

Gonopods relatively large, of the form illustrated in figures 4–7. Sternite large and subtriangular, not firmly attached to the coxites, latter unusual in that the outer (lateral) paragonocoel is enlarged and folded strongly mesad at the expense of the inner member so that the gonocoel opens on the caudal side of the coxite. Latter distally bilobed, forming a partially closed notch through which the telopodite passes. Paracoxites large, flattened, about half as long as paragonocoel. Telocoxite completely lacking terminal structure beyond end of gonocoel. Prostatic stylet large, thick, distally flared (fig. 5).

Telopodite bent abruptly proximad and closely appressed to the anterior face of the coxite before becoming distally broadened and hyaline-laminate. Femoral portion slender and unmodified, set off from the tibiotarsal portion by a distinct suture, no femoral spine ("kniedorn" of Attems) present. Tibiotarsal region becoming abruptly very broad, thin, and laminate, coiled into two complete circles and much more compact than as shown in the drawings which were made from a slide mount of gonopods from a paratype. One edge of the tibiotarsal blade is ornamented with a dentate hyaline fringe which begins proximally from a large rounded lobe. Distal end of telopodite drawn out into a long, slender solenomerite, subtended by a large subsagitate marginal lobe (fig. 6).

Remarks: In many of the preserved specimens examined, the male gonopods were completely extruded from the 7th segment, with a much greater frequency than observed in other series of spirostreptoids. Perhaps many of these millipeds were collected while mating, but there are no mated pairs preserved in amplexus.

Cook's rather brief couplets in his key to the genera of the Trachystreptidae (2) accurately describe material of *cambaloides* (as regards external appearance), although *Trachystreptus* is the third genus eliminated. Cook's keys were constructed in such a way as to account for genera or species in the decreasing order of their distinctness: the most unusual forms going out first. The last two genera of Trachystreptidae in the key, *Ptilostreptus* and *Lophostreptus*, had to be distinguished by some rather subjective differences—doubtless only specific in value—and the two names have long since been considered synonymous by Count Attems (4).

The family Trachystreptidae was admittedly founded solely upon the segmental ornamentation of its species, Cook remarking the similarity of its genera to typical spirostreptoids in most other respects. Yet even within the genera which he considered to be trachystreptoids, there is obviously considerable structural diversity. I have been able to compare specimens only of two species of *Lophostreptus*, but to my mind these species differ as much from *T. cambaloides* as they do from normal spirostreptoids. Apparently the Kamerun species *Lemostreptus tuberculosus* (Porat), which I know only from the original description, is even more extremely modified than is *Trachystreptus*. I suspect that the Trachystreptidae in the sense of Cook is actually an assemblage of unrelated forms, possibly random survivors of an ancestral cambaloid-spirostreptoid stock. Whether the name Trachystreptidae is to be retained for future use would seem to depend entirely upon characters of the gonopods, particularly the curious form of the coxites. Such an evaluation can scarcely be made until the genitalia of all genera of the typical spirostreptoids have been studied from the standpoint of comparative morphology.

ACKNOWLEDGMENT

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Interactions of Ergot Strains in Saprophytic Culture

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Recent advances in the investigation of the ergot fungus in saprophytic culture have shown that it is possible to obtain sizeable yields of clavine alkaloids under certain conditions; however, the saprophytic production of large quantities of the peptide alkaloids has not been achieved (1). The therapeutic importance of the latter group of compounds prompted a re-evaluation of the theories explaining the biosynthesis of the ergoline and lysergic acid derivatives, the ultimate objective being the saprophytic production of peptide-type alkaloids.

Voigt (2) has reviewed the literature relating to the sequence of formation of the clavine and peptide-type ergot alkaloids. He summarized the possibilities essentially as follows:

1. **Precursor→Clavine Alkaloids→Ergonovine→Peptide-Type Alkaloids**

2. **Precursor→Clavine Alkaloids** ↗ **Ergonovine**
   Clavine Alkaloids ↘ **Peptide-Type Alkaloids**

3. **Precursor→** ↗ **Clavine Alkaloids**
   Ergonovine ↘ **Peptide-Type Alkaloids**

Abe (3) has postulated that an aldehyde derivative of lysergic acid was the common starting point for two metabolic pathways, one of which led to the ergoline derivatives and the other to lysergic acid and subsequently the peptide-type alkaloids.

On the basis of the postulated biosynthetic relationships between the clavine alkaloids and the lysergic acid derivatives it was considered desirable to study strains of *Claviceps*, capable of producing peptide-type alkaloids parasitically, grown in mixed culture with high-yielding, clavine-producing strains. If the clavine alkaloids are intermediates in the biosynthesis of the lysergic acid derivatives, or if the clavine-producing strains form an aldehyde intermediate which would diffuse into the culture medium and if the necessary enzyme systems were present in the mycelium of the peptide-type strain, quantities of peptide-type alkaloids should be formed.

EXPERIMENTAL METHODS AND RESULTS

Selection of Ergot.—Quantitative and qualitative analyses of ten samples of wild grass ergot, one of barley ergot, one sample of ergot from Tetra Petkus rye, and a commercial sample of Ergot NF were made to give a basis for selecting the strains for saprophytic study. The quantitative data were obtained essentially by the procedure of Silber and Schulze (4). Duplicate analyses were carried out with each sample. The fat content was determined by allowing the petroleum ether extracts in the tared Soxhlet flasks to evaporate in a forced-air oven at 45° C and calculating the weight of the fatty residue after storage in a desiccator over anhydrous calcium chloride. The absorbancies of the colored solutions

[1]This manuscript is abstracted in part from a dissertation submitted to the Graduate School of the University of Washington by C. I. Abou-Chaar in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: American University of Beirut, Beirut, Lebanon.

resulting from the ultraviolet irradiation of the alkaloid solutions plus the *p*-dimethylaminobenzaldehyde reagent were measured in a Bausch and Lomb Spectronic 20 colorimeter at 590 mμ. The quantities of alkaloids were calculated from a standard curve prepared with ergonovine maleate, USP Reference Standard and expressed on the basis of the dried, defatted sclerotia. The average quantitative data are listed in table 1.

Ammoniacal ether extracts of additional portions of the dried, defatted sclerotia were prepared and concentrated to a volume of 1 ml for each 100 mg of drug. Quantities of 10–30 μl of the concentrated extracts together with solutions of known peptide-type alkaloids were chromatographed in duplicate by the procedure of Meinicke (5). The identity and relative concentration of the peptide-type alkaloids in each sample were determined by visual examination of the chromatograms under filtered ultraviolet radiation and are recorded in table 2.

TABLE 1. *Results of quantitative analyses of ergot sclerotia.*

Sample	Host	Collection data	Fat %	Water-soluble alkaloids[1] %	Water-insoluble alkaloids[2] %
22N60	*Agropyron smithii* Rydb.	Hall County, Nebr. August 1960	10.91±0.10	0.027±0.000	0.718±0.002
3N60	*Bromus inermis* Leyss.	Hall County, Nebr. July 1960	13.20±0.10	0.031±0.004	0.401±0.006
1N59	*Elymus canadensis* L.	Buffalo County, Nebr. August 1959	8.31[3]	0.034±0.003	1.179±0.031
2N59	*E. canadensis* L.	Buffalo County, Nebr. August 1959	9.15±0.18	0.035±0.001	0.945±0.011
25N60	*E. canadensis* L.	Hall County, Nebr. August 1960	10.90±0.76	0.035±0.002	1.029±0.033
34N60	*E. canadensis* L.	Buffalo County, Nebr. August 1960	8.92±0.08	0.046±0.001	1.024±0.038
1S60	*E. mollis* Trin.	Camano Island, Wash. September 1960	18.37±0.10	0.020±0.002	0.592±0.009
19N60	*E. virginicus* L.	Buffalo County, Nebr. July 1960	11.45±0.09	0.037±0.000	0.985±0.006
26N60	*E. virginicus* L.	Hall County, Nebr. August 1960	8.63±0.17	0.043±0.005	1.155±0.007
40N60	*E. virginicus* L.	Hall County, Nebr. September 1960	8.05±0.04	0.038±0.001	1.246±0.003
JW60	*Hordeum vulgare* L.	Obtained from Dr. J. L. Weihing, Nebr.	12.67±0.06	0.031±0.000	0.432±0.002
TP60	*Secale cereale* L. (Tetra Petkus)	Obtained from Dr. J. L. Weihing, Nebr.	13.21±0.02	0.022±0.002	0.382±0.009
NF	*S. cereale* L.	Commercial Ergot NF	19.69±0.03	0.026±0.001	0.345±0.003

[1]Calculated as ergonovine (mol wt 325).
[2]Calculated as ergotamine (mol wt 582).
[3]A portion of one of the solutions was spilled, and the corresponding value was discarded.

Investigation of Stationary Mixed Saprophytic Cultures.—Clavine-producing strains 15B and 47A were grown in mixed cultures with ergot strains 1N59, 2N59 and three additional strains. Two of the latter strains were *Claviceps purpurea* (Fries) Tul. which had been isolated from sclerotia parasitic upon *Holcus lanatus* L. collected on Vancouver Island, British Columbia, in September 1960 (2V60) and upon *Lolium perenne* L. collected in King County, Washington, in September 1960 (5W60), respectively. The third strain was isolated from the sclerotia of *C. paspali* Stevens and Hall collected from *Paspalum dilatatum* Poir. in Arkansas during the summer of 1960 (1P60).

Isolates of the various ergot strains were prepared from the respective sclerotia

as described by Tyler (6). When sufficient mycelial development had been obtained on the agar slants, transfers of the organisms were made to 500-ml Erlenmeyer flasks containing 100 ml each of a sterile, modified Stoll-Abe nutrient medium (normal nutrient medium) of the following composition: mannitol, 50 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 0.013 g; $ZnSO_4 \cdot 7H_2O$, 0.004 g; $(NH_4)_2SO_4$, 3.668 g; succinic acid, 5.4 g; and distilled water, 1 liter. The *p*H of the medium was adjusted to 5.2 with 20 per cent potassium hydroxide solution. These flasks were placed on a New Brunswick rotary shaker, model V, operating at 200 rpm until a thick mycelial growth was obtained. These stock cultures were stored in a refrigerator until inoculum was needed.

The ergot strains were grown singly and in various combinations (table 3) in stationary culture in duplicate sets of 1-liter Roux-type culture bottles containing 125 ml each of one of two different media. One of the media was the normal nutrient medium, and the other was Brady and Tyler's nutrient medium no. 2 with the sucrose-low phosphate modifications (7). Strain 15B and its combinations were grown only in the normal medium since previous results had shown that it was not well adapted to the sucrose-low phosphate medium (7).

In the mixed cultures it was necessary to restrict the growth of the mycelium of a given strain to approximately one-half of the available surface area but

TABLE 2. *Identity and relative concentrations of the water-insoluble alkaloids in the ergot sclerotia.*

Sample	Ergotamine	Ergosine	Ergocristine-Ergocornine	Ergocryptine
22N60	+++	+	+	++
3N60	++	++	+	+
1N59	+++	++	+	+
2N59	+++	++	++	+
25N60	++	++	+	++
34N60	+++	++	++	++
1S60	+++	+	++	+
19N60	++	+	+++	+++
26N60	++	++	+	++
40N60	++	+	+++	+++
JW60	++	−	+	trace
TP60	+	+	+++	+

to allow an interchange of metabolites. This was effected by inserting glass wool dams into the culture bottles. A piece of glass wool was rolled into a tight cylinder about 1 cm in diameter, tied with glass wool thread, cut into lengths of 12.5 cm, and inserted in the appropriate Roux bottles before sterilization of the medium. Prior to inoculation the glass wool cylinders were adjusted with an inoculating needle so that the surfaces of the media within the bottles were divided into nearly equal parts, one toward the mouth of the bottle and the other toward the base. One strain of ergot was introduced behind the glass wool divider and the other in front of it; the order of inoculation was reversed in each of the pairs of mixed cultures. After two weeks in a constant temperature cabinet at 25° C both strains had surfaced in all cases, and the relative abundance of mycelium of each strain was noted. Each bottle was then tilted gently back and forth to promote effective mixing of the medium on both sides of the partition. The cultures were returned to the constant temperature cabinet for further incubation.

Small samples of the respective media were aseptically decanted on the thirty-fifth day following inoculation, and the alkaloid content of each sample was determined by an established procedure (6). The cultures were allowed to

incubate until the fortieth day at which time the mycelial mats were separated from the media. The mycelium from each Roux bottle was collected on a small, wire-mesh strainer, rinsed with about 400 ml of distilled water, placed in a porcelain dish, and dried in a forced-air oven at 45° C for five days. The mycelial mats were then placed in a desiccator over anhydrous calcium chloride for twenty-four hours, weighed to the nearest centigram, and stored for further analysis. The alkaloid content of the medium was determined as before, and 20–150 μl quantities

TABLE 3. *Alkaloids accumulated in the media of saprophytic cultures and their identity.*

Ergot strains	Age of culture days	Alkaloid content[1] in normal medium μg/ml	Alkaloid content[1] in sucrose-low phosphate medium μg/ml	Identity of alkaloids[2] in normal medium	Identity of alkaloids[2] in sucrose-low phosphate medium
47A	35	24.0± 0.0	479.5±47.5	E,A,P,S	E,A,P,S
	40	18.0± 0.0	552.5±68.5		
15B	35	103.0± 3.0	———	A	———
	40	101.0± 4.0	———		
1N59	35	14.5± 1.5	15.5± 0.5	none	none
	40	16.0± 1.0	16.0± 0.0		
1N59+47A	35	11.5± 1.5	56.0± 5.0	none	E,A,P,S
	40	14.5± 0.5	53.5± 3.5		
1N59+15B	35	17.5± 1.5	———	A,S	———
	40	17.5± 1.5	———		
2N59	35	10.0± 1.0	20.0[3]	none	A,E
	40	12.0± 2.0	20.0[3]		
2N59+47A	35	20.0± 3.0	98.5± 7.5	E	E,A,P,S
	40	20.0± 3.0	101.5± 2.5		
2N59+15B	35	25.0± 2.0	———	A,S	———
	40	20.5± 1.5	———		
2V60	35	10.0± 0.0	11.5± 0.5	none	none
	40	10.0± 0.0	11.5± 0.5		
2V60+47A	35	16.0± 3.0	10.0± 0.0	E,A,P,S	none
	40	16.0± 4.0	11.0± 0.0		
2V60+15B	35	34.5±17.5	———	A,S	———
	40	30.5±15.5	———		
5W60	35	8.0± 0.0	11.5± 0.5	none	none
	40	8.5± 0.5	14.0± 0.0		
5W60+47A	35	10.0± 0.0	10.5± 0.5	E	none
	40	10.5± 0.5	12.5± 0.5		
5W60+15B	35	18.5± 3.5	———	A,S	———
	40	17.5± 3.5	———		
1P60	35	0.0± 0.0	0.0± 0.0	none	none
	40	0.0± 0.0	0.0± 0.0		
1P60+47A	35	———	48.0±12.0	———	E,P
	40	———	49.5±16.5		
1P60+15B	35	35.0±15.0	———	A,S	———
	40	25.0±10.0	———		

[1]Average results of duplicate cultures calculated as agroclavine (mol wt 238).
[2]*A* agroclavine; *E* elymoclavine; *P* penniclavine; and *S* setoclavine. Arranged in decreasing order of abundance.
[3]One of the culture flasks became contaminated and was discarded.

of the respective media were spotted on buffered sheets of Whatman no. 1 filter paper and chromatographed with water-saturated *n*-butanol (6) to establish the qualitative nature of the alkaloids. The results are reported in table 3.

In certain cases the data in table 3 pertaining to the quantitative and qualitative analyses appear to be inconsistent. The quantitative procedure necessarily measured total indole compounds, including tryptophan and other non-alkaloid

derivatives. Hence, qualitative data concerning the presence of alkaloids are required for the evaluation of media with low quantitative results.

When the presence of alkaloids was doubtful, ammoniacal ether extracts or ammoniacal ethanol extracts of the culture media were prepared. Twenty ml of the respective culture media which were rendered alkaline with 0.8 ml of diluted ammonium hydroxide solution were extracted in a separatory funnel with three successive 10-ml portions of ether. The combined ether extracts were evaporated to a volume of 0.5 ml by a current of air. The ethanolic extracts were prepared by evaporating a 20-ml portion of the respective media to dryness under reduced pressure, extracting the resulting residue with three successive 2 ml portions of ethanol which contained 4 per cent of diluted ammonium hydroxide solution, combining the ethanolic extracts, centrifuging at 1750 rpm for thirty minutes, evaporating the supernatant liquid to a 0.5-ml volume under reduced pressure, centrifuging again, and transferring the clear supernatant solution to a dry test

TABLE 4. *Data from the analyses of the mycelial mats from the stationary cultures.*

Ergot strains	Mycelial weight (ave) from normal medium g	Mycelial weight (ave) from sucrose-low phosphate medium g	Alkaloid content[1] in mycelium from normal medium μg/g	Alkaloid content[1] in mycelium from sucrose-low phosphate medium μg/g	Alkaloid identity[2] from normal medium	Alkaloid identity[2] from sucrose-low phosphate medium
47A	2.08±0.00	2.82±0.08	261.0± 11.0	3262.0± 48.0	E,A,P,S	E,A,P,S
15B	2.12±0.03	———	1148.0± 4.0	———	A	——
1N59	1.37±0.01	2.07±0.16	0.0± 0.0	0.0± 0.0	none	none
1N59+47A	1.51±0.04	2.23±0.16	75.0± 2.0	162.0± 29.0	E	E,A,P,S
1N59+15B	1.53±0.07	———	614.0± 24.0	———	A,S	——
2N59	1.18±0.03	2.01[3]	0.0± 0.0	0.0± 0.0	none	none
2N59+47A	1.53±0.22	2.17±0.04	221.0± 43.0	297.0± 11.0	E,P	E,A,P,S
2N59+15B	1.48±0.07	———	837.0±171.0	———	A,S	——
2V60	1.69±0.00	2.04±0.03	0.0± 0.0	0.0± 0.0	none	none
2V60+47A	1.24±0.06	2.02±0.00	156.0± 88.0	0.0± 0.0	E,P,A,S	none
2V60+15B	1.49±0.09	———	545.0±285.0	———	A,S	——
5W60	1.18±0.03	1.76±0.11	0.0± 0.0	0.0± 0.0	none	none
5W60+47A	0.92±0.07	1.94±0.23	0.0± 0.0	0.0± 0.0	none	none
5W60+15B	1.40±0.05	———	488.0±199.0	———	A,S	——
1P60	2.01±0.09	1.58±0.08	0.0± 0.0	0.0± 0.0	none	none
1P60+47A	———	1.89±0.22	———	596.0±315.0	——	E,P,A.S
1P60+15B	1.78±0.08	———	615.0± 74.0	———	A,S	——

[1]Average results of duplicate cultures calculated as agroclavine (mol wt 238).

[2]*A* agroclavine; *E* elymoclavine; *P* penniclavine; *S* setoclavine. Arranged in decreasing order of abundance.

[3]One of the culture flasks became contaminated and was discarded.

tube. Quantities of 30–50 μl of the respective extracts were spotted on filter paper impregnated with buffer at *p*H 5.0 and chromatographed with water-saturated *n*-butanol (6). The ether or ethanol extracts were also spotted and chromatographed by the method of Meinicke (5) to identify any peptide-type alkaloids which might be present.

The dried mycelial mats from the stationary cultures were milled to a no. 60 powder and maintained for twenty-four hours over anhydrous calcium chloride. Aliquots of 0.1 to 0.17 g were assayed by a slight modification of the Silber and Schulze method which was used for the sclerotia. The mycelium was not defatted, and the total alkaloids were extracted directly from the ammoniacal ether extract with 2 per cent tartaric acid solution. The tartaric acid solution was assayed. Ammoniacal ether extracts prepared from additional aliquots of the dried mycelia were reduced to 1 ml volumes, and 30 to 50 μl quantities were chromatographed

by the same procedures which were used for the extracts of the media. The results are reported in table 4.

A visual comparison of the color intensity and spot size of the respective alkaloids when the various chromatograms were prepared with equivalent volumes of the alkaloid extracts of mixed cultures containing strain 47A indicated a relative decrease in the concentration of elymoclavine and an increase in the concentration of penniclavine. This phenomenon was especially noticeable in the media from the cultures of 2V60+47A in normal nutrient medium and of 1N59+47A, 2N59+47A, and 1P60+47A in the sucrose-low phosphate medium. This observation was also noted for the mycelia from cultures of 2N59+47A and 2V60+47A grown in the normal medium and of 1N59+47A, 2N59+47A, and 1P60+47A grown in the sucrose-low phosphate medium. A comparable situation was observed in the mixed cultures involving strain 15B inasmuch as setoclavine appeared in the older cultures, and the amount of agroclavine appeared to decrease slightly; however, this was not as pronounced as the previous change. This observation coupled with the relatively high alkaloid content of the mixed mycelia from cultures involving strain 15B led to the conclusion that the alkaloid producing capacity of this clavine-producing strain is comparatively stable.

Three additional *p*-dimethylaminobenzaldehyde-positive spots were detected on certain of the chromatograms, the most interesting of which occurred in highest concentration in the sucrose-low phosphate medium from cultures of 1N59+47A and 2N59+47A and which was identified subsequently as lysergic acid (8). A substance was observed in the sucrose-low phosphate medium from the cultures of 1P60+47A which had a violet fluorescence under ultraviolet radiation, developed a yellow color with the *p*-dimethylaminobenzaldehyde reagent, and exhibited R_F 0.13 on buffered paper with water-saturated *n*-butanol. The R_F resembled that of kynurenine, but this relationship was not substantiated upon further study. The chromatograms of the mycelial extracts from the cultures of 2V60+47A and 5W60+47A grown in the sucrose-low phosphate medium revealed a substance with R_F 0.75–0.80 on the buffered paper with the *n*-butanol solvent system; this substance gave a strong fluorescence with ultraviolet radiation and a greenish-yellow color when sprayed with the *p*-dimethylaminobenzaldehyde reagent.

No peptide-type alkaloids were detected in any of the culture media or mycelia grown singly or in the various mixed cultures. However, a small amount of agroclavine and a trace of elymoclavine were detected in the medium when strain 2N59 was grown singly on the sucrose-low phosphate medium.

Investigation of Submerged Saprophytic Cultures.—It is well established that the metabolism of the ergot fungus varies, at least quantitatively and in rate, in submerged culture. Hence, a preliminary study was conducted to test whether the formation of peptide-type alkaloids could be induced by placing a preformed mycelium of a given strain with peptide-type alkaloid-producing potential in a clavine-rich medium.

Strain SD/58 which is known to form agroclavine, chanoclavine, and elymoclavine in appropriate media was employed as the clavine-producing component. Two different strains which have been used in Europe in the agricultural production of ergot sclerotia were used as the strains with peptide-type alkaloid-producing potential. Strain XXIV produces an alkaloid fraction consisting of 90 per cent ergotamine and 10 per cent other, mixed alkaloids parasitically, and strain XXXIII yields an alkaloid fraction consisting of 50 per cent ergocornine and 50 per cent ergocryptine.

The clavine-containing medium was prepared by inoculating a 2800-ml Fernbach flask which contained 350 ml of NL406 medium plus 20 ml of 1,2-propanediol with strain SD/58. Medium NL406 had the following composition: mannitol, 50 g; sucrose, 50 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 0.01 g;

$ZnSO_4 \cdot 7H_2O$, 0.0044 g; succinic acid, 5.4 g; yeast extract, 1 g; and distilled water, 1 liter. The *p*H of the medium was adjusted to 5.4 with 25 per cent ammonium hydroxide solution. This culture was shaken for four days on a Bar-Nun rotary flask agitator, model M, size no. 30-2, operating at 200 rpm, at which time the alkaloid content of the medium was 200 μg/ml. The mycelium was separated from the medium by filtering through a sintered-glass funnel under aseptic conditions. The mycelium was discarded, and 50-ml portions of the alkaloid-containing nutrient medium were transferred to each of six sterile, 500-ml Erlenmeyer flasks.

The mycelia of the peptide-type alkaloid-producing strains were prepared by inoculating in duplicate 500-ml Erlenmeyer flasks, containing 100 ml of NL406 medium each, with strains XXIV and XXXIII respectively. The cultures were placed on the Bar-Nun agitator for fifteen days at which time abundant mycelia had been formed. The respective pairs of the submerged cultures were combined, the media separated by suction filtration under aseptic conditions, and the mycelia thoroughly washed with sterile water. The washed mycelia were suspended in small volumes of sterile water, and 5 ml of the inoculating suspension of strains XXIV or XXXIII were used to inoculate each flask of the clavine alkaloid-containing medium. Five ml of the inoculating suspensions of strains XXIV and XXXIII corresponded to 0.150 g and 0.210 g of dried mycelium respectively.

TABLE 5. *Data from the analyses of submerged cultures.*

Ergot strain	Culture age days	Mycelial weight g	Alkaloid content[1] of medium μg/ml	Alkaloids remaining in medium %[2]	Identity of alkaloids[3] in medium
SD/58	0	——	200	100.0	E,A,C
XXIV	3	0.200	173	86.5	E,A,C,S
XXXIII	3	0.200	173	86.5	E,A,C,S
XXIV	5	0.295	190	91.2	E,A,C,S
XXXIII	5	0.350	177	88.5	E,A,C,S
XXIV	10	0.370	185	88.8	E,A,C,S,P
XXXIII	10	0.320	182	91.0	E,A,C,S,P

[1]Calculated as agroclavine (mol wt 238).

[2]A final volume of 50 ml was assumed for purposes of calculation. The actual volume was determined to vary from 48 to 50 ml.

[3]*A* agroclavine; *C* chanoclavine; *E* elymoclavine; *P* penniclavine; *S* setoclavine. Arranged in decreasing order of abundance.

The flasks were placed on the shaker and removed after three, five and ten days of incubation; the alkaloid content was determined by the method of Silber and Schulze. The qualitative nature of the alkaloids was established by the thin-layer chromatographic system no. I of Gröger, *et al.* (9). The results are reported in table 5.

The data revealed an approximate 12 per cent decrease in total alkaloid content in the medium. Setoclavine appeared after three days and penniclavine was detected in the medium after ten days of incubation, suggesting a pattern similar to that observed in the mixed stationary cultures. No observable difference was noted between the two peptide-type strains with regard to alkaloid composition, and the formation of peptide-type alkaloids was not induced under the experimental conditions.

DISCUSSION

The observation that peptide-type alkaloids were not present when various ergot strains with peptide-type alkaloid-producing potential were grown in the presence of clavine alkaloids does not necessarily validate or invalidate the postu-

lations that the clavine alkaloids are precursors of the lysergic acid derivatives or that an aldehyde derivative of lysergic acid is the precursor of the peptide-type alkaloids. Lack of sufficient information concerning a number of factors precludes a conclusive solution to this problem at the present time. For example, no data are available regarding the ability of the mycelium to absorb the clavine alkaloids from the medium, regarding the endo- or exocellular accumulation of significant quantities of the hypothetical lysergic aldehyde, or as to the ability of such a substance to pass through the cell walls of the mycelium. Likewise, basic knowledge regarding the presence of the necessary enzymes and/or cofactors in the saprophytic mycelium of ergot strains which will form peptide-type alkaloids parasitically is lacking.

The decrease in the amounts of agroclavine and elymoclavine and a simultaneous increase in the amounts of setoclavine and penniclavine respectively during the incubation period in this investigation suggest certain biosynthetic relationships. A comparison of the chemical structures of the two pairs of compounds reveals that such conversions could occur via oxidative metabolism, and the observation is consistent with hypothetical pathways which have been noted by Abe (3). Recently, Agurell and Ramstad (10) established one of these pathways in strain 47A when they reported the conversion of C^{14}-labeled elymoclavine to radioactive penniclavine. Penniclavine and lysergic acid both have a double bond in the 9–10 position, and it is conceivable that the lysergic acid which was detected in the present investigation was derived from penniclavine through a two-step oxidation. The data reported in this paper, a theoretical consideration of previous hypotheses, and the agroclavine→elymoclavine→penniclavine sequence which has been established (10) permit a reorientation of the various components into the following hypothetical scheme:

↗Setoclavine
Agroclavine→Elymoclavine→Penniclavine→(Lysergic Aldehyde)→

Lysergic acid→Peptide-Type Alkaloids.

This postulation emphasizes that the formation of lysergic acid *per se* in saprophytic cultures is no indication that peptide-type ergot alkaloids will be formed. The lack of formation of peptide-type alkaloids in this investigation in the cultures which formed lysergic acid, and the observation by Gjerstad (11) that added lysergic acid was not metabolized by *Claviceps*, experimentally confirm this absence of an obligatory relationship. The basis for such a metabolic block could vary depending upon such factors as the absence of an essential component in an enzyme system, or a physical separation of the substrate and the enzyme. The experimental data in this investigation fail to indicate why the lysergic acid was not converted to the peptide-type alkaloids.

SUMMARY

Samples of ergot sclerotia collected from seven different grasses were analyzed in order to obtain cultures of alkaloid-rich strains. Sclerotia from *Elymus canadensis* and *Elymus virginicus* were found which contained 0.034–0.046 and 0.945–1.246 per cent of water-soluble and water-insoluble alkaloids respectively. However, when these, or similar strains, were grown in various combinations with clavine alkaloid-producing strains or in replacement cultures containing clavine alkaloids, no peptide-type alkaloids were formed. One strain, 2N59 from *E. canadensis*, did produce a small amount of agroclavine and a trace of elymoclavine when it was grown saprophytically on a sucrose-low phosphate medium.

A decrease in the accumulation of clavine alkaloids by strains 15B and 47A was observed in their respective mixed cultures. A less pronounced decrease in

the total amount of clavine alkaloids was noted when the preformed mycelia of two different strains were placed in a clavine-rich medium.

Experimental data suggest that elymoclavine is converted to penniclavine and agroclavine to setoclavine. This evidence, coupled with the agroclavine→elymoclavine→penniclavine sequence established by Agurell and Ramstad and with a consideration of previous biosynthetic hypotheses, permit construction of the following hypothetical sequence for ergot alkaloid biosynthesis:

$$\begin{array}{l} \nearrow \textbf{Setoclavine} \\ \textbf{Agroclavine} \rightarrow \textbf{Elymoclavine} \rightarrow \textbf{Penniclavine} \rightarrow (\textbf{Lysergic Aldehyde}) \rightarrow \\ \textbf{Lysergic acid} \rightarrow \textbf{Peptide-Type Alkaloids.} \end{array}$$

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A New Species of Kernia

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Four coprophilous eurotiaceous species which have at one time or another been placed in the genus *Magnusia* appear to belong in the genus *Kernia*.[1]

A new species was found on dead leaves of *Artocarpus heterophyllus* in 1959. Culture tubes planted with dead leaves of *A. heterophyllus* produced light-yellow colonies which later developed appendaged cleistothecia. The species differs from others in its habitat and morphology. The non-septate cleistothecial appendages, which form a perfect spiral, are frequently branched and thus differ from the closely coiled appendages of *K. spirotricha*, and apically circinate appendages of *K. brachytricha*, the copius elevated clusters of elongate appendages of *K. nitida* and the very flexuous narrow appendages of *K. bartlettii*. In none of the above species are the asci cylindrical but the new species has very long and cylindrical asci. The size of the ascospores is also slightly larger. The present form is considered to be a new species and is described as follows:

Kernia furcotricha sp. n.

Cleistothecia juvenilia pallide brunnea, matura vero nigra, superficialia, vulgo globosa, 143–187.2×137.8–150 μ operta capillie longis flexuosis non-septatis 1.3–1.5 μ latis, 162–190 μ longis; plurimae appendices furcatae spirales etiam adornant parietes cleistothecii, capillis spiralibus convolutionibus usque 14 ornatis; appendices plene extensae usque ad 260 μ longae, ca. 2.6 μ latae. Asci plurimi, in catervas corymboideas dispositi absque paraphysibus, hyalini, cylindrici, 44.2–52×8.3 μ ascospous octo. Ascosporae hyalinae, primo, tandem pallide brunneae, acutae ad unum apicem. Inficit folia *Artocarpi heterophylli*.

Type collected by K. S. Bilgrami, Allahabad Agricultural Institute, Naini, July 7, 1959. The type (a culture) is deposited in Commonwealth Mycological Institute, Kew, England.

Cleistothecia olive brown when young and black at maturity, superficial, generally globose (fig. 1, A), measuring 143–187.2×137.8–150 μ, covered with long flexuous hairs which are generally uniformly distributed, 1.3–1.5 μ in diameter and 162–190 μ in length. A large number of furcate spiral appendages also adorn the cleistothecial wall (fig. 1, D) some of them may be up to 14 turns with a maximum width of 2.6 μ and the length when fully stretched reaching up to 260 μ. Asci numerous in corymboid clusters, lacking paraphyses, hyaline and cylindrical, 44.2–52×8.3 μ with light obliquely arranged ascospores (fig. 1, B). Ascospores hyaline when young becoming light brown at maturity, acute at one end (fig. 1, C). Conidial stage not observed. Ascospores 7.5–11× 6.5 μ

[1] *Kernia nitida* (Sacc.) Nieuw.
Kernia bartlettii (Mass. and Sal.), comb. nov. (*Magnusia bartletti* Mass. and Sal., Ann. Botan. **15:** 333. 1901).
Kernia brachytricha (Ames), comb. nov. (*Magnusia brachytricha* Ames, Mycologia **29:** 223. 1937).
Kernia spirotricha (Benjamin), comb. nov. (*Magnusia spirotricha* Benjamin, El Aliso **3:** 199. 1955).

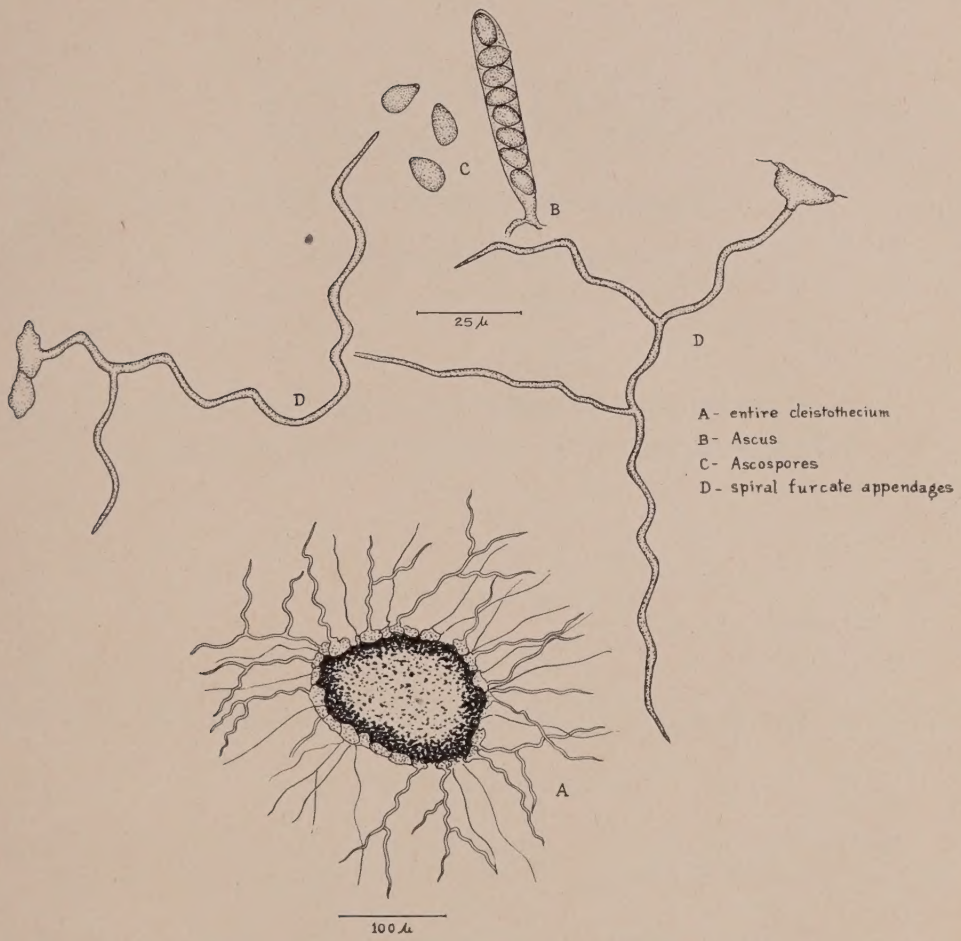

FIG. 1. **Kernia furcotricha** *sp. n.*

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